Nucleic acid sequences encoding proteins associated with abiotic stress response

This invention relates generally to nucleic acid sequences encoding proteins that are associated with abiotic stress responses and abiotic stress tolerance in plants. In particular, this invention relates to nucleic acid sequences encoding proteins that confer drought, heat, cold, and/or salt tolerance to plants.

Abiotic environmental stresses, such as drought stress, salinity stress, heat stress, and cold stress, are major limiting factors of plant growth and productivity (Boyer. 1982. *Science* 218, 443–448). Crop losses and crop yield losses of major crops such as rice, maize (corn) and wheat caused by these stresses represent a significant economic and political factor and contribute to food shortages in many underdeveloped countries.

Plants are typically exposed during their life cycle to conditions of reduced environmental water content. Most plants have evolved strategies to protect themselves against these conditions of low water or desiccation (drought). However, if the severity and duration of the drought conditions are too great, the effects on plant development, growth and yield of most crop plants are profound. Continuous exposure to drought causes major alterations in the plant metabolism. These great changes in metabolism ultimately lead to cell death and consequently yield losses.

Developing stress-tolerant plants is a strategy that has the potential to solve or mediate at least some of these problems (McKersie and Leshem, 1994. *Stress and Stress Coping in Cultivated Plants*, Kluwer Academic

Publishers). However, traditional plant breeding strategies to develop new lines of plants that exhibit resistance (tolerance) to these types of stresses are relatively slow and require specific resistant lines for crossing with the desired line. Limited germplasm resources for stress tolerance and incompatibility in crosses between distantly related plant species represent significant problems encountered in conventional breeding. Additionally, the cellular processes leading to drought, cold and salt tolerance are complex in nature and involve multiple mechanisms of cellular adaptation and numerous metabolic pathways (McKersie and Leshem, 1994. *Stress and Stress Coping in Cultivated Plants*, Kluwer Academic Publishers). This multi-component nature of stress tolerance has not only made breeding for tolerance largely unsuccessful, but has also limited the ability to genetically engineer stress tolerance plants using biotechnological methods.

Drought, heat, cold and salt stresses have a common theme important for plant growth and that is water availability. Plants are exposed during their entire life cycle to conditions of reduced environmental water content. Most plants have evolved strategies to protect themselves against these conditions. However, if the severity and duration of the drought conditions are too great, the effects on plant development, growth and yield of most crop plants are profound. Since high salt content in some soils result in less available water for cell intake, its effect is similar to those observed under drought conditions. Additionally, under freezing temperatures, plant cells loose water as a result of ice formation that starts in the apoplast and withdraws water from the symplast (McKersie and Leshem, 1994. *Stress and Stress Coping in Cultivated Plants*, Kluwer Academic Publishers). Commonly, a plant's molecular response mechanisms to each of these stress conditions are common.

The results of current research indicate that drought tolerance is a complex quantitative trait and that no real diagnostic marker is available yet. High salt concentrations or dehydration may cause damage at the cellular level during drought stress but the precise injury is not entirely clear (Bray, 1997. *Trends Plant Sci.* 2, 48–54). This lack of a mechanistic understanding makes it difficult to design a transgenic approach to improve drought tolerance. However, an important consequence of damage may be the production of reactive oxygen radicals that cause cellular injury, such as lipid peroxidation or protein and nucleic acid modification. Details of oxygen free radical chemistry and their reaction with cellular components such as cell membranes have been described (McKersie and Leshem, 1994. *Stress and Stress Coping in Cultivated Plants*, Kluwer Academic Publishers).

There are numerous sites of oxygen activation in the plant cell, which are highly controlled and tightly coupled to prevent release of intermediate products (McKersie and Leshem, 1994. *Stress and Stress Coping in Cultivated Plants*, Kluwer Academic Publishers). Under abiotic stress situations, it is likely that this control or coupling breaks down and the process "dysfunctions" leaking activated oxygen. These uncoupling events are not detrimental provided that they are short in duration and that the oxygen scavenging systems are able to detoxify the various forms of activated oxygen. If the production of activated oxygen exceeds the plant's capacity to detoxify it, deleterious degenerative reactions occur. At the subcellular level, disintegration of membranes and aggregation of proteins are typical symptoms. Therefore it is the balance between the production and the scavenging of activated oxygen that is critical to the maintenance of active growth and metabolism of the plant and overall environmental (abiotic) stress tolerance.

Preventing or diminishing the accumulation of oxygen free radicals in response to drought is a potential way to engineer tolerance (Allen, 1995.

Plant Physiol. 107, 1049–1054). Overexpression of antioxidant enzymes or ROS-scavenging enzymes is one possibility for the induction of functional detoxification systems. For example, transgenic alfalfa plants expressing Mn-superoxide dismutase tend to have reduced injury after water-deficit stress (McKersie et al., 1996. Plant Physiol. 111, 1177–1181). These same transgenic plants have increased biomass production in field trials (McKersie et al., 1999. Plant Physiology, 119: 839–847; McKersie et al., 1996. Plant Physiol. 111, 1177–1181). Transgenic plants that overproduce osmolytes such as mannitol, fructans, proline or glycine-betaine also show increased resistance to some forms of abiotic stress and it is proposed that the synthesized osmolytes act as ROS scavengers (Tarczynski. *et al.* 1993. . Science 259, 508–510; Sheveleva,. *et al.* 1997. Plant Physiol.115, 1211–1219). Overexpression of glutathione reductase has increased antioxidant capacity and reduced photoinhibition in popular trees (Foyer et al., 1995. Plant Physiology 109: 1047-57).

The glutaredoxin and thioredoxin proteins are small heat-stable oxidoreductases that have been conserved throughout evolution. They function in many cellular processes, including deoxyribonucleotide synthesis, protein folding, sulfur metabolism and most notably repair of oxidatively damaged proteins. They have also been implicated in the regulation of redox homeostasis in the cell and redox potential has been implicated in changes in gene expression.

Thioredoxins have a dithiol/disulfide (CGPC) at their active site and are the major cellular protein disulfide reductases. Cytosolic isoforms are present in most organisms. Mitochondria have a separate thioredoxin system and plants have chloroplast thioredoxins, which regulate photosynthetic enzymes by light via ferredoxin-thioredoxin reductase. Thioredoxins are critical for redox regulation of protein function and signaling via thiol redox control.

Several transcription factors require thioredoxin reduction for DNA binding (Arner and Holmgren, 2000. European Journal of Biochemistry 267: 6102-6109; Spyrou et al., 2001. Human Genetics 109: 429-439).

Glutaredoxins are small heat-stable proteins that are active as glutathione-dependent oxidoreductases. They catalyze glutathione-disulfide oxidoreductions overlapping the functions of thioredoxins and using reducing equivalents from NADPH via glutathione reductase. In *Saccharomyces cerevisiae*, two genes, GRX1 and GRX2, whose expression is induced in response to various stress conditions including oxidative, osmotic, and heat stress, encode glutaredoxins. Furthermore, both genes are activated by the high-osmolarity glycerol pathway and negatively regulated by the Ras-protein kinase (Grant CM. 2001. Molecular Microbiology 39: 533-541; Grant CM et al.,. 2001. Biochimica et Biophysica Acta - Gene Structure & Expression 1490: 33-42).

Another subfamily of yeast glutaredoxins (Grx3, Grx4, and Grx5) differs from the first in containing a single cysteine residue at the putative active site (Rodriguez-Manzaneque et al., 1999. Molecular & Cellular Biology 19: 8180-8190). The role of these enzymes is not fully understood.

In addition to the two gene pairs encoding cytoplasmic glutaredoxins (GRX1, GRX2), Saccharomyces cerevisiae also contains two gene pairs for thioredoxins (TRX1, TRX2). Only a quadruple mutant is non-viable and either a single glutaredoxin or a single thioredoxin can sustain viability, indicating some cross function between the two systems (Draculic et al., 2000. Molecular Microbiology 36: 1167-1174).

Plants also contain glutaredoxins genes. A glutaredoxin (thioltransferase), which catalyzes thiol/disulfide exchange reaction, was isolated from rice (Oryza saliva L.) (Sha et al., 1997. Journal of Biochemistry 121: 842-848; Sha et al., 1997. Gene 188: 23-28; GenBank accession

number D86744). Mulitple forms of glutaredoxin have also been predicted in the Arabiposis genome (GenBank).

Dehydroascorbate reductase (DHAR; glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1) is an enzyme that is critical for maintenance of an appropriate level of ascorbate in plant cells by the cycling of dehydroascorbate to replenish ascorbate. DHAR was considered a specific enzyme of the ascorbate-glutathione cycle. However, at least four distinct proteins can catalyze *in vitro* both glutathione-dependent DHA reduction and other reactions mainly related to thiol-disulphide exchange. These glutaredoxin enzymes (thioltransferases) have both thiol-disulfide oxidoreductase and dehydroascorbate reductase activities (Kato et al., 1997. Plant & Cell Physiology 38: 173-178; Detullio et al., 1998. Plant Physiology & Biochemistry 36: 433-440). Therefore glutaredoxins may also function *in vivo* as DHAR.

There have been no reports on the mutation or overexpression of either thioredoxin or glutaredoxin in plant cells to determine their function in terms of oxidative stress tolerance or drought tolerance.

It is the object of this invention to identify new, unique genes capable of conferring stress tolerance to plants upon over-expression.

The present invention provides a transgenic plant cell transformed by Oxidoreductase Stress-Related Protein (ORSRP) coding nucleic acid, wherein expression of the nucleic acid sequence in the plant cell results in increased tolerance and/or resistance to environmental stress as compared to a corresponding non-transformed wild type plant cell. One preferred wild type plant cell is a non-transformed Arabidopsis plant cell. An example here is the

Arabidopsis wild type C24 (Nottingham Arabidopsis Stock Centre, UK ; NASC Stock N906).

Preferably the oxidoreductase stress related protein is heat-stable. The invention provides that the environmental stress can be salinity, drought, temperature, metal, chemical, pathogenic and oxidative stresses, or combinations thereof.

The object of the invention is a transgenic plant cell, wherein the ORSRP is heat-stable. Further,

in said transgenic plant cell, the ORSRP is selected from yeast or plant. Preferably, in a transgenic plant of the instant invention, the ORSRP is selected from the group comprising glutaredoxin and/or thioredoxin protein. Further the invention pertains to a transgenic plant cell, wherein the ORSRP coding nucleic acid is selected from the group comprising SEQ ID No. 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants and/or homologs thereof. Object of the invention is also a transgenic plant cell, wherein the ORSRP coding nucleic acid is at least about 50 % homologous to SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49.

The invention further provides a seed produced by a transgenic plant transformed by a ORSRP coding nucleic acid, wherein the plant is true breeding for increased tolerance to environmental stress as compared to a wild type plant cell. The transgenic plant might be a monocot, a dicot or a gymnosperm plant. The invention further provides a seed produced by a transgenic plant expressing an ORSRP wherein the plant is true breeding for increased tolerance to environmental stress as compared to a wild type plant cell.

The invention further provides an agricultural product produced by any of the below-described transgenic plants, plant parts such as leafs, roots, stems, buds, flowers or seeds. The invention further provides a isolated recombinant expression vector comprising a ORSRP encoding nucleic acid.

The invention further provides a method of producing a transgenic plant with a ORSRP coding nucleic acid, wherein expression of the nucleic acid in the plant results in increased tolerance to environmental stress as compared to a wild type plant comprising: (a) transforming a plant cell with an expression vector comprising a ORSRP coding nucleic acid, and (b) generating from the plant cell a transgenic plant with an increased tolerance to environmental stress as compared to a wild type plant.

With regard to invention described here, "transgenic or transgene" means all those plants or parts thereof which have been brought about by genetic manipulation methods and in which either

a) the nucleic acid sequence as depicted in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 or a homologe thereof, or

b) a genetic regulatory element, for example a promoter, which is functionally linked to the nucleic acid sequence as depicted in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 or a homologe thereof, or

c) (a) and (b)

is/are not present in its/their natural genetic environment or has/have been modified by means of genetic manipulation methods, it being possible for the modification to be, by way of example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide radicals. "Natural genetic environment" means the natural chromosomal locus in the organism

of origin or the presence in a genomic library. In the case of a genomic library, the natural, genetic environment of the nucleic acid sequence is preferably at least partially still preserved. The environment flanks the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp, preferably at least 500 bp, particularly preferably at least 1000 bp, very particularly preferably at least 5000 bp.

In said method, the used ORSRP is heat-stable. Further, the ORSRP used in the instant method described above is a glutaredoxin or thioredoxin protein. Herein the ORSRP coding nucleic acid is selected from the group comprising SEQ ID No. 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants and/or homologs thereof. Further, the ORSRP coding nucleic acid used in the said method is at least about 50% homologous to SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49.

A plant or plant cell is considered "true breeding" for a particular trait if it is genetically homozygous for that trait to the extent that, when the true-breeding plant is self-pollinated, a significant amount of independent segregation of the trait among the progeny is not observed. In the present invention, the trait arises from the transgenic expression of one or more DNA sequences introduced into a plant cell or plant.

The present invention also provides methods of modifying stress tolerance of a plant comprising, modifying the expression of a ORSRP nucleic acid in the plant. The invention provides one method of producing a transgenic plant with a synthetic, novel or modified transcription factor that acts by increasing or decreasing the transcription of a ORSRP gene.

The present invention also provides methods of modifying stress tolerance of a crop plant comprising utilizing a ORSRP coding nucleic acid sequence to identify individual plants in populations segregating for either increased or decreased environmental stress tolerance (DNA marker).
In the said method of modifying stress tolerance of a plant the ORSRP encoding nucleic acid is selected from the group comprising SEQ ID No. 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants and/or homologs thereof. Further the ORSRP coding nucleic acid used therein is at least about 50% homologous to SEQ ID No. SEQ ID No. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49. Also an expression vector as described in the instant invention might be used in the said method.
In an variant method of said method of modifying stress tolerance, the plant is transformed with an inducible promoter that directs expression of the ORSRP. For example, the promoter is tissue specific. In a variant method, the used promoter is developmentally regulated.
In the instant method of modifying stress tolerance in plant the ORSRP expression is modified by administration of an antisense molecule and/or by double stranded RNA interference that inhibits expression of ORSPR. In another variant of the method, ORSRP expression is modified by administration of an targeting nucleic sequence complementary to the regulatory region of the ORSRP encoding nucleic acid and/or by a transcription factor and/or by a zinc finger protein.

The present invention relates to a method for the identification of loci for stress tolerance phenotypes in individual plants. Genomic regions associated with environmental stress tolerance can be identified using Quantitative Trait Loci (QTL) mapping analysis. This approach may use either variation in the

glutaredoxin or thioredoxin nucleic acid sequence, variation in the surrounding genomic sequences or variation in the expression level of glutaredoxin or thioredoxin nucleic acid sequence as the quantitative trait.

The invention provides that the above methods can be performed such that the stress tolerance is either increased or decreased.

This invention is not limited to specific nucleic acids, specific polypeptides, specific cell types, specific host cells, specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art. It is also to be understood that the terminology used herein is for the purpose of describing specific embodiments only and is not intended to be limiting.

The present invention describes that particularly glutaredoxin or thioredoxin genes are useful for increasing a plant's tolerance and/or resistance to environmental stress. Accordingly, the present invention provides glutaredoxin and thioredoxin gene sequences selected from the group consisting of SEQ ID No. 1, 3, 5, 7, 9, 11, 13 from *Saccharomyces cerevisiae*.

This invention provides sequences of glutaredoxin and thioredoxin nucleic acids that are responsive to drought and environmental conditions in *Brassica napus*, *Arabidopsis thaliana* and *Oryza sativa* according to SEQ ID 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 and that exhibit homology at the nucleic acid and amino acid level to the yeast genes in SEQ ID 3 and 7, respectively. These plant homologs are functionally equivalent according to this invention to yeast genes of SEQ ID 3 and 7 and can be used to provide environmental stress tolerance in plants.

The invention also pertain to an isolated Oxidoreductase Stress Related Protein (ORSRP) which is selected form the group comprising SEQ ID No. 16, 18, 20, 22, 24, 44 and 50. Further the isolated Oxidoreductase Stress Related Protein (ORSRP) as mentioned before is heat-stable. The isolated Oxidoreductase Stress Related Protein (ORSRP) selected form the group comprising SEQ ID No. 16, 18, 20, 22, 24, 44 and 50 is selected form plant. Preferred is an isolated Oxidoreductase Stress Related Protein (ORSRP) selected form the group comprising SEQ ID No. 16, 18, 20, 22, 24, 44 and 50 wherein the ORSRP is a glutaredoxin or thioredoxin protein.

Another object of the instant invention is an isolated Oxidoreductase Stress Related Protein (ORSRP) encoding nucleic acid selected from the group comprising SEQ ID No. 15, 17, 19, 21, 23, 45 and 49. Said isolated Oxidoreductase Stress Related Protein (ORSRP) encoding nucleic acid encoding an ORSRP which is heat-stable. Thereby the isolated Oxidoreductase Stress Related Protein (ORSRP) encoding nucleic acid selected from the group comprising SEQ ID No. 15, 17, 19, 21, 23, 45 and 49 encoding an ORSRP which is selected from plants. Preferred is an isolated Oxidoreductase Stress Related Protein (ORSRP) encoding nucleic acid selected from the group comprising SEQ ID No. 15, 17, 19, 21, 23, 45 and 49 wherein the ORSRP is a glutaredoxin or thioredoxin .

Homologs of the aforementioned sequences can be isolated advantageously from yeast, fungi or plants, preferably from yeasts such as from the genera Saccharomyces, Pichia, Candida, Hansenula, Torulopsis or Schizosaccharomyces, or plants such as Arabidopsis thaliana, maize, wheat, rye, oat, triticale, rice, barley, soybean, peanut, cotton, borage, sufflower, linseed, primrose, rapeseed, canola and turnip rape, manihot, pepper,

sunflower, tagetes, solanaceous plant such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa, bushy plants such as coffee, cacao, tea, Salix species, trees such as oil palm, coconut, perennial grass, such as ryegrass and fescue, and forage crops, such as alfalfa and clover and from spruce, pine or fir for example, more preferably from Saccharomyces cerevisiae or plants.

The glutaredoxin or thioredoxin of the present invention are preferably produced by recombinant DNA techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector, for example in to a binary vector, the expression vector is introduced into a host cell, for example the Arabidopsis thaliana wild type NASC N906 or any other plant cell as described in the examples see below, and the glutaredoxin or thioredoxin is expressed in said host cell. Examples for binary vectors are pBIN19, pBI101, pBinAR, pGPTV or pPZP (Hajukiewicz, P. et al., 1994, plant Mol. Biol., 25: 989-994).

As used herein, the term "environmental stress" refers to any sub-optimal growing condition and includes, but is not limited to, sub-optimal conditions associated with salinity, drought, temperature, metal, chemical, pathogenic and oxidative stresses, or combinations thereof. In preferred embodiments, the environmental stress can be salinity, drought, heat, or low temperature, or combinations thereof, and in particular, can be low water content or low temperature. Wherein drought stress means any environmental stress which leads to a lack of water in plants or reduction of water supply to plants, wherein low temperature stress means freezing of plants below + 4 °C as well as chilling of plants below 15 °C and wherein high temperature stress means for example a temperature above 35 °C. The

range of stress and stress response depends on the different plants which are used for the invention, i.e. it differs for example between a plant such as wheat and a plant such as Arabidopsis. It is also to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

As also used herein, the terms "nucleic acid" and "nucleic acid molecule" are intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. This term also encompasses untranslated sequence located at both the 3' and 5' ends of the coding region of the gene: at least about 1000 nucleotides of sequence upstream from the 5' end of the coding region and at least about 200 nucleotides of sequence downstream from the 3' end of the coding region of the gene. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one that is substantially separated from other nucleic acid molecules, which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of some of the sequences that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated glutaredoxin or thioredoxin nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be free from

some of the other cellular material with which it is naturally associated, or culture medium when produced by recombinant techniques, or chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule encoding an ORSRP or a portion thereof which confers tolerance and/or resistance to environmental stress in plants, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, a *Arabidopsis thaliana* glutaredoxin or thioredoxin cDNA can be isolated from a *A. thaliana* library using all or portion of one of the sequences of SEQ ID 1, 3, 5, 7, 9, 11, 13 of yeast. Moreover, a nucleic acid molecule encompassing all or a portion of one of the sequences of SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon this sequence. For example, mRNA can be isolated from plant cells (e.g., by the guanidinium-thiocyanate extraction procedure of Chirgwin et al., 1979 Biochemistry 18:5294-5299) and cDNA can be prepared using reverse transcriptase (e.g., Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for polymerase chain reaction amplification can be designed based upon one of the nucleotide sequences shown in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast. A nucleic acid molecule of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid molecule so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to a glutaredoxin or thioredoxin nucleotide

sequence can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises one of the nucleotide sequences shown in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants encoding the glutaredoxin or thioredoxin (i.e., the "coding region"), as well as 5' untranslated sequences and 3' untranslated sequences.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the coding region of one of the sequences in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants, for example, a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of a glutaredoxin or thioredoxin.

Portions of proteins encoded by the glutaredoxin or thioredoxin nucleic acid molecules of the invention are preferably biologically active portions of one of the glutaredoxin or thioredoxin described herein. As used herein, the term "biologically active portion of" a glutaredoxin or thioredoxin is intended to include a portion, e.g., a domain/motif, of a glutaredoxin or thioredoxin that participates in a stress tolerance and/or resistance response in a plant. To determine whether a glutaredoxin or thioredoxin , or a biologically active portion thereof, results in increased stress tolerance in a plant, a stress analysis of a plant comprising the glutaredoxin or thioredoxin may be performed. Such analysis methods are well known to those skilled in the art, as detailed in the Examples. More specifically, nucleic acid fragments encoding biologically active portions of a glutaredoxin or thioredoxin can be prepared by isolating a portion of one of the sequences in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37,

39, 41, 43, 45, 47, 49 of plants, expressing the encoded portion of the glutaredoxin or thioredoxin or peptide (e.g., by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the glutaredoxin or thioredoxin or peptide.

Biologically active portions of a glutaredoxin or thioredoxin are encompassed by the present invention and include peptides comprising amino acid sequences derived from the amino acid sequence of a glutaredoxin or thioredoxin gene, or the amino acid sequence of a protein homologous to a glutaredoxin or thioredoxin, which include fewer amino acids than a full length glutaredoxin or thioredoxin or the full length protein which is homologous to a glutaredoxin or thioredoxin, and exhibits at least some enzymatic activity of a glutaredoxin or thioredoxin. Typically, biologically active portions (e.g., peptides which are, for example, 5, 10, 15, 20, 30, 35, 36, 37, 38, 39, 40, 50, 100 or more amino acids in length) comprise a domain or motif with at least one activity of a glutaredoxin or thioredoxin enzyme. Moreover, other biologically active portions in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the activities described herein. Preferably, the biologically active portions of a glutaredoxin or thioredoxin include one or more selected domains/motifs or portions thereof having biological activity.

In addition to fragments of the glutaredoxin or thioredoxin described herein, the present invention includes homologs and analogs of naturally occurring glutaredoxin or thioredoxin and glutaredoxin or thioredoxin encoding nucleic acids in a plant.

"Homologs" are defined herein as two nucleic acids or proteins that have similar, or "homologous", nucleotide or amino acid sequences, respectively. Homologs include allelic variants, orthologs, paralogs, agonists and antagonists of glutaredoxin or thioredoxin as defined hereafter. The term

"homolog" further encompasses nucleic acid molecules that differ from one of the nucleotide sequences shown in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants (and portions thereof) due to degeneracy of the genetic code and thus encode the same glutaredoxin or thioredoxin as that encoded by the amino acid sequences shown in SEQ ID No. 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50 of plants. As used herein a "naturally occurring" glutaredoxin or thioredoxin refers to a glutaredoxin or thioredoxin amino acid sequence that occurs in nature.

Moreover, nucleic acid molecules encoding glutaredoxin or thioredoxin from the same or other species such as glutaredoxin or thioredoxin analogs, orthologs and paralogs, are intended to be within the scope of the present invention. As used herein, the term "analogs" refers to two nucleic acids that have the same or similar function, but that have evolved separately in unrelated organisms. As used herein, the term "orthologs" refers to two nucleic acids from different species that have evolved from a common ancestral gene by speciation. Normally, orthologs encode proteins having the same or similar functions. As also used herein, the term "paralogs" refers to two nucleic acids that are related by duplication within a genome. Paralogs usually have different functions, but these functions may be related (Tatusov, R.L. et al. 1997 Science 278(5338):631-637). Analogs, orthologs and paralogs of a naturally occurring glutaredoxin or thioredoxin can differ from the naturally occurring glutaredoxin or thioredoxin by post-translational modifications, by amino acid sequence differences, or by both. Post-translational modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation, and such modifications may occur during polypeptide synthesis

or processing or following treatment with isolated modifying enzymes. In particular, orthologs of the invention will generally exhibit at least 80-85%, more preferably 90%, and most preferably 95%, 96%, 97%, 98% or even 99% identity or homology with all or part of a naturally occurring glutaredoxin or thioredoxin amino acid sequence and will exhibit a function similar to a glutaredoxin or thioredoxin. Orthologs of the present invention are also preferably capable of participating in the stress response in plants.

In addition to naturally-occurring variants of a glutaredoxin or thioredoxin sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into a nucleotide sequence of SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants, thereby leading to changes in the amino acid sequence of the encoded glutaredoxin or thioredoxin, without altering the functional ability of the glutaredoxin or thioredoxin. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in a sequence of SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of one of the glutaredoxin or thioredoxin s without altering the activity of said glutaredoxin or thioredoxin , whereas an "essential" amino acid residue is required for glutaredoxin or thioredoxin activity. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved in the domain having glutaredoxin or thioredoxin activity) may not be essential for activity and thus are likely to be amenable to alteration without altering glutaredoxin or thioredoxin activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding glutaredoxin or thioredoxin that contain changes in

amino acid residues that are not essential for glutaredoxin or thioredoxin activity. Such glutaredoxin or thioredoxin differ in amino acid sequence from a sequence comprising of SEQ IDs 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 ,48, 50 of plants, yet retain at least one of the glutaredoxin or thioredoxin activities described herein. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 50% homologous to an amino acid sequence of SEQ IDs 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 ,48, 50. Preferably, the protein encoded by the nucleic acid molecule is at least about 50-60% homologous to one of the sequences of SEQ ID No. 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50 of plants, more preferably at least about 60-70% homologous to one of the sequences of SEQ ID No. 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 ,48, 50 of plants, even more preferably at least about 70-80%, 80-90%, 90-95% homologous to one of the sequences of SEQ ID No. 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 ,48, 50 of plants, and most preferably at least about 96%, 97%, 98%, or 99% homologous to one of the sequences of SEQ IDs 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 ,48, 50 of plants. The preferred glutaredoxin or thioredoxin homologs of the present invention are preferably capable of participating in the stress tolerance response in a plant. The homology (= identity) was calculated over the entire amino acid range. The program used was PileUp (J. Mol. Evolution., 25 (1987), 351-360, Higgins et al., CABIOS, 5 1989: 151-153).

Variants shall also be encompassed, in particular, functional variants which can be obtained from the sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants by means of deletion, insertion or substitution of nucleotides, the enzymatic activity of the derived synthetic proteins being retained.

An isolated nucleic acid molecule encoding a glutaredoxin or thioredoxin homologous to a protein sequence of SEQ IDs 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 ,48, 50 of plants can be created by introducing one or more nucleotide substitutions, additions or deletions into a nucleotide sequence of SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into one of the sequences of SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain.

Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine,

tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a glutaredoxin or thioredoxin is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a glutaredoxin or thioredoxin coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for a glutaredoxin or thioredoxin activity described herein to identify mutants that retain glutaredoxin or thioredoxin activity. Following mutagenesis of one of the sequences of SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants, the encoded protein can be expressed recombinantly and the activity of the protein can be determined by analyzing the stress tolerance of a plant expressing the protein as described in Examples below.

In addition to the nucleic acid molecules encoding the glutaredoxin or thioredoxin described above, another aspect of the invention pertains to isolated nucleic acid molecules that are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence that is complementary to a "sense" nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire glutaredoxin or thioredoxin coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding a glutaredoxin or thioredoxin . The term "coding region" refers to the region of the nucleotide sequence comprising codons that are translated into amino

acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding a glutaredoxin or thioredoxin . The term "noncoding region" refers to 5' and 3' sequences that flank the coding region that are not translated into amino acids (i.e., also referred to as 5' and 3' untranslated regions).

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of one of the nucleotide sequences shown in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants, or a portion thereof. A nucleic acid molecule that is complementary to one of the nucleotide sequences shown in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants is one which is sufficiently complementary to one of the nucleotide sequences shown in SEQ IDs 3 or 7such that it can hybridize to one of these nucleotide sequences, thereby forming a stable duplex.

Given the coding strand sequences encoding the glutaredoxin or thioredoxin disclosed herein (e.g., the sequences set forth in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of glutaredoxin or thioredoxin mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of glutaredoxin or thioredoxin mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of glutaredoxin or thioredoxin mRNA.

An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 or more nucleotides in length.

It is also possible to use the inverted repeat technology combining an antisense fragment with a portion of the antisense fragment in sense orientation linked by either an adapter sequence or an excisable intron (Abstract Book of the 6th Intern. Congr. Of Plant Mol. Biol. ISPMB, Quebec June 18-24,2000, Abstract No. S20-9 by Green et al.).

An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v),

5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a cell or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a glutaredoxin or thioredoxin to thereby inhibit expression of the protein, e.g., by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. The antisense molecule can be modified such that it specifically binds to a receptor or an antigen expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecule to a peptide or an antibody which binds to a cell surface receptor or antigen. The antisense nucleic acid molecule can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong prokaryotic, viral, or eukaryotic (including plant) promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier et al., 1987 Nucleic Acids. Res. 15:6625-6641). The antisense

nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al., 1987 Nucleic Acids Res. 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue et al., 1987 FEBS Lett. 215:327-330).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (e.g., hammerhead ribozymes described in Haselhoff and Gerlach, 1988 Nature 334:585-591) can be used to catalytically cleave glutaredoxin or thioredoxin mRNA transcripts to thereby inhibit translation of glutaredoxin or thioredoxin mRNA. A ribozyme having specificity for a glutaredoxin or thioredoxin -encoding nucleic acid can be designed based upon the nucleotide sequence of a glutaredoxin or thioredoxin cDNA, as disclosed herein (i.e., SEQ IDs 1-76) or on the basis of a heterologous sequence to be isolated according to methods taught in this invention. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a glutaredoxin or thioredoxin -encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071 and Cech et al. U.S. Patent No. 5,116,742. Alternatively, glutaredoxin or thioredoxin mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel, D. and Szostak, J.W., 1993 Science 261:1411-1418.

Another embodiment of the invention is the regulating of the glutaredoxin or thioredoxin genes by means of double-stranded RNA ("double-stranded RNA interference"; dsRNAi) which has been described repeatedly for animal and plant organisms (for example Matzke MA et al. (2000) Plant Mol Biol 43:401-415; Fire A. et al (1998) Nature 391:806-811;

WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035; WO 00/63364). Express reference is made to the processes and methods described in the above references. Such effective gene suppression can for example also be demonstrated upon transient expression or following transient transformation for example as the consequence of biolistic transformation (Schweizer P et al. (2000) Plant J 2000 24: 895-903). dsRNAi methods are based on the phenomenon that the simultaneous introduction of complementary strand and counterstrand of a gene transcript causes the expression of the gene in question to be suppressed in a highly efficient manner. The phenotype caused greatly resembles a corresponding knock-out mutant (Waterhouse PM et al. (1998) Proc Natl Acad Sci USA 95:13959-64).

As described, inter alia, in WO 99/32619, dsRNAi approaches are markedly superior to traditional antisense approaches.

The invention therefore furthermore relates to double-stranded RNA molecules (dsRNA molecules) which, upon introduction into a plant (or a cell, tissue, organ or seed derived therefrom), bring about the reduction of an glutaredoxin or thioredoxin gene. In the double-stranded RNA molecule for reducing the expression of an glutaredoxin or thioredoxin protein,

a) one of the two RNA strands is essentially identical to at least a portion of an glutaredoxin or thioredoxin nucleic acid sequence, and

b) the corresponding other RNA strand is essentially identical to at least a portion of the complementary strand of an glutaredoxin or thioredoxin nucleic acid sequence.

"Essentially identical" means that the dsRNA sequence can also show insertions, deletions or individual point mutations compared with the

glutaredoxin or thioredoxin target sequence while still bringing about an effective reduction of the expression. The homology in accordance with the above definition preferably amounts to at least 75%, preferably at least 80%, very especially preferably at least 90%, most preferably 100%, between the sense strand of an inhibitory dsRNA and a part-segment of an glutaredoxin or thioredoxin nucleic acid sequence (or between the antisense strand and the complementary strand of an glutaredoxin or thioredoxin nucleic acid sequence). The length of the part-segment amounts to at least 10 bases, preferably at least 25 bases, especially preferably at least 50 bases, very especially preferably at least 100 bases, most preferably at least 200 bases or at least 300 bases. As an alternative, an "essentially identical" dsRNA can also be defined as a nucleic acid sequence which is capable of hybridizing with part of an glutaredoxin or thioredoxin gene transcript (for example in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 505C or 705C for 12 to 16 h).

The dsRNA can be composed of one or more strands of polymerized ribonucleotides. Modifications both of the sugar-phosphate backbone and of the nucleosides may be present. For example, the phosphodiester bonds of the natural RNA can be modified in such a way that they comprise at least one nitrogen or sulfur hetero atom. Bases can be modified in such a way that the activity of, for example, adenosine deaminase is restricted.

The dsRNA can be generated enzymatically or fully or partially synthesized chemically.

The double-stranded structure can be formed starting from an individual self-complementary strand or starting from two complementary strands. In a single self-complementary strand, sense and antisense sequence may be linked by a linking sequence ("linker") and can form for

example a hairpin structure. The linking sequence can preferably be an intron which is spliced out after the dsRNA has been synthesized. The nucleic acid sequence encoding a dsRNA can comprise further elements such as, for example, transcription termination signals or polyadenylation signals. If the two dsRNA strands are to be combined in a cell or plant, this can be effected in various ways:

a) transformation of the cell or plant with a vector comprising both expression cassettes,

b) cotransformation of the cell or plant with two vectors, one of them comprising the expression cassettes with the sense strand and the other comprising the expression cassettes with the antisense strand,

c) hybridizing two plants, each of which has been transformed with one vector, one of the vectors comprising the expression cassettes with the sense strand and the other comprising the expression cassettes with the antisense strand.

The formation of the RNA duplex can be initiated either outside or within the cell. Like in WO 99/53050, the dsRNA can also encompass a hairpin structure by linking sense and antisense strand by means of a linker (for example an intron). The self-complementary dsRNA structures are preferred since they only require the expression of one construct and always comprise the complementary strands in an equimolar ratio.

The expression cassettes encoding the antisense or sense strand of a dsRNA or the self-complementary strand of the dsRNA are preferably inserted into a vector and, using the methods described herein, stably inserted into the genome of a plant in order to ensure permanent expression of the dsRNA, using selection markers for example.

The dsRNA can be introduced using a quantity which allows at least one copy per cell. Greater quantities (for example at least 5, 10, 100, 500 or 1000 copies per cell) may bring about a more effective reduction.

As already described, 100% sequence identity between dsRNA and an glutaredoxin or thioredoxin gene transcript is not necessarily required in order to bring about an effective reduction of the glutaredoxin or thioredoxin expression. Accordingly, there is the advantage that the method is tolerant with regard to sequence deviations as may exist as the consequence of genetic mutations, polymorphisms or evolutionary divergence. Thus, for example, it is possible to use the dsRNA generated on the basis of the glutaredoxin or thioredoxin sequence of one organism to suppress the glutaredoxin or thioredoxin expression in another organism. The high sequence homology between the glutaredoxin or thioredoxin sequences from different sources allows the conclusion that this protein is conserved to a high degree within plants, so that the expression of a dsRNA derived from one of the disclosed glutaredoxin or thioredoxin sequences as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 of yeast and/or SEQ ID No. 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46 ,48, 50 of plants appears to have an advantageous effect in other plant species as well.

The dsRNA can be synthesized either in vivo or in vitro. To this end, a DNA sequence encoding a dsRNA can be brought into an expression cassette under the control of at least one genetic control element (such as, for example, promoter, enhancer, silencer, splice donor or splice acceptor or polyadenylation signal). Suitable advantageous constructions are described herein. Polyadenylation is not necessarily required, nor do elements for initiating translation have to be present.

A dsRNA can be synthesized chemically or enzymatically. Cellular RNA polymerases or bacteriophage RNA polymerases (such as, for example,

T3, T7 or SP6 RNA polymerase) can be used for this purpose. Suitable methods for expression of RNA in vitro are described (WO 97/32016; US 5,593,874; US 5,698,425, US 5,712,135, US 5,789,214, US 5,804,693). A dsRNA which has been synthesized in vitro chemically or enzymatically can be isolated completely or to some degree from the reaction mixture, for example by extraction, precipitation, electrophoresis, chromatography or combinations of these methods, before being introduced into a cell, tissue or organism. The dsRNA can be introduced directly into the cell or else be applied extracellularly (for example into the interstitial space).

However, it is preferred to transform the plant stably with an expression construct which brings about the expression of the dsRNA. Suitable methods are described herein.The methods of dsRNAi, cosuppression by means of sense RNA and "VIGS" ("virus induced gene silencing") are also termed "post-transcriptional gene silencing" (PTGS). PTGS methods, like the reduction of the glutaredoxin or thioredoxin function or activity with dominant-negative glutaredoxin or thioredoxin variants, are especially advantageous because the demands regarding the homology between the endogenous gene to be suppressed and the sense or dsRNA nucleic acid sequence expressed recombinantly (or between the endogenous gene and its dominant-negative variant) are lower than, for example, in the case of a traditional antisense approach. Such criteria with regard to homology are mentioned in the description of the dsRNAi method and can generally be applied to PTGS methods or dominant-negative approaches. Owing to the high degree of homology between the glutaredoxin or thioredoxin proteins from different sources, a high degree of conservation of this protein in plants can be assumed. Thus, using the glutaredoxin or thioredoxin nucleic acid sequences from yeast, it is presumably also possible efficiently to suppress the expression of homologous glutaredoxin or

thioredoxin proteins in other species such as plants without the isolation and structure elucidation of the glutaredoxin or thioredoxin homologs occurring therein being required. Considerably less labor is therefore required.

All of the substances and compounds which directly or indirectly bring about a reduction in protein quantity, RNA quantity, gene activity or protein activity of an glutaredoxin or thioredoxin protein shall subsequently be combined in the term "anti- glutaredoxin or thioredoxin " compounds. The term "anti- glutaredoxin or thioredoxin " compound explicitly includes the nucleic acid sequences, peptides, proteins or other factors employed in the above-described methods.

For the purposes of the invention, "introduction" comprises all of the methods which are capable of directly or indirectly introducing an "anti-glutaredoxin or thioredoxin " compound into a plant or a cell, compartment, tissue, organ or seed thereof, or of generating such a compound there. Direct and indirect methods are encompassed. The introduction can lead to a transient presence of an "anti- glutaredoxin or thioredoxin " compound (for example a dsRNA) or else to its stable presence.

Alternatively, glutaredoxin or thioredoxin gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of a glutaredoxin or thioredoxin nucleotide sequence (e.g., a glutaredoxin or thioredoxin promoter and/or enhancer) to form triple helical structures that prevent transcription of a glutaredoxin or thioredoxin gene in target cells. See generally, Helene, C., 1991 Anticancer Drug Des. 6(6):569-84; Helene, C. et al., 1992 Ann. N.Y. Acad. Sci. 660:27-36; and Maher, L.J., 1992 Bioassays 14(12):807-15.

In particular, a useful method to ascertain the level of transcription of the gene (an indicator of the amount of mRNA available for translation to the gene product) is to perform a Northern blot (for reference see, for example,

Ausubel et al., 1988 Current Protocols in Molecular Biology, Wiley: New York). This information at least partially demonstrates the degree of transcription of the transformed gene. Total cellular RNA can be prepared from cells, tissues or organs by several methods, all well-known in the art, such as that described in Bormann, E.R. et al., 1992 Mol. Microbiol. 6:317-326. To assess the presence or relative quantity of protein translated from this mRNA, standard techniques, such as a Western blot, may be employed. These techniques are well known to one of ordinary skill in the art. (See, for example, Ausubel et al., 1988 Current Protocols in Molecular Biology, Wiley: New York).

The invention further provides an isolated recombinant expression vector comprising a Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin nucleic acid as described above, wherein expression of the vector or glutaredoxin or thioredoxin nucleic acid, respectively in a host cell results in increased tolerance and/or resistance to environmental stress as compared to the wild type of the host cell. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the

expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

A plant expression cassette preferably contains regulatory sequences capable of driving gene expression in plant cells and operably linked so that each sequence can fulfill its function, for example, termination of transcription by polyadenylation signals. Preferred polyadenylation signals are those originating from *Agrobacterium tumefaciens* T-DNA such as the gene 3 known as octopine synthase of the Ti-plasmid pTiACH5 (Gielen et al., 1984 EMBO J. 3:835) or functional equivalents thereof but also all other terminators functionally active in plants are suitable.

As plant gene expression is very often not limited on transcriptional levels, a plant expression cassette preferably contains other operably linked sequences like translational enhancers such as the overdrive-sequence containing the 5'-untranslated leader sequence from tobacco mosaic virus enhancing the protein per RNA ratio (Gallie et al., 1987 Nucl. Acids Research 15:8693-8711).

Plant gene expression has to be operably linked to an appropriate promoter conferring gene expression in a timely, cell or tissue specific manner. Preferred are promoters driving constitutive expression (Benfey et al., 1989 EMBO J. 8:2195-2202) like those derived from plant viruses like the 35S CaMV (Franck et al., 1980 Cell 21:285-294), the 19S CaMV (see also U.S. Patent No. 5352605 and PCT Application No. WO 8402913) or plant

promoters like those from Rubisco small subunit described in U.S. Patent No. 4,962,028.

Additional advantageous regulatory sequences are, for example, included in the plant promoters such as CaMV/35S [Franck et al., Cell 21 (1980) 285 - 294], PRP1 [Ward et al., Plant. Mol. Biol. 22 (1993)], SSU, OCS, lib4, usp, STLS1, B33, LEB4, nos or in the ubiquitin, napin or phaseolin promoter. Also advantageous in this connection are inducible promoters such as the promoters described in EP-A-0 388 186 (benzyl sulfonamide inducible), Plant J. 2, 1992: 397 - 404 (Gatz et al., Tetracyclin inducible), EP-A-0 335 528 (abscisic acid inducible) or WO 93/21334 (ethanol or cyclohexenol inducible). Additional useful plant promoters are the cytosolic FBPase promotor or ST-LSI promoter of the potato (Stockhaus et al., EMBO J. 8, 1989, 2445), the phosphorybosyl phyrophoshate amido transferase promoter of Glycine max (gene bank accession No. U87999) or the noden specific promoter described in EP-A-0 249 676. Additional particularly advantageous promoters are seed specific promoters which can be used for monokotyledones or dikotyledones are described in US 5,608,152 (napin promoter from rapeseed), WO 98/45461 (phaseolin promoter from Arobidopsis), US 5,504,200 (phaseolin promoter from Phaseolus vulgaris), WO 91/13980 (Bce4 promoter from Brassica), Baeumlein et al., Plant J., 2, 2, 1992: 233-239 (LEB4 promoter from leguminosa) said promoters are useful in dikotyledones. The following promoters are useful for example in monokotyledones lpt-2- or lpt-1- promoter from barley (WO 95/15389 and WO 95/23230), hordein promoter from barley and other useful promoters described in WO 99/16890.

It is possible in principle to use all natural promoters with their regulatory sequences like those mentioned above for the novel process. It is also possible and advantageous in addition to use synthetic promoters.

The gene construct may also comprise further genes which are to be inserted into the organisms and which are for example involved in stress resistance. It is possible and advantageous to insert and express in host organisms regulatory genes such as genes for inducers, repressors or enzymes which intervene by their enzymatic activity in the regulation, or one or more or all genes of a biosynthetic pathway. These genes can be heterologous or homologous in origin. The inserted genes may have their own promoter or else be under the control of same promoter as the sequences SEQ ID No. 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants or their homologs.

The gene construct advantageously comprises, for expression of the other genes present, additionally 3' and/or 5' terminal regulatory sequences to enhance expression, which are selected for optimal expression depending on the selected host organism and gene or genes.

These regulatory sequences are intended to make specific expression of the genes and protein expression possible as mentioned above. This may mean, depending on the host organism, for example that the gene is expressed or overexpressed only after induction, or that it is immediately expressed and/or overexpressed.

The regulatory sequences or factors may moreover preferably have a beneficial effect on expression of the introduced genes, and thus increase it. It is possible in this way for the regulatory elements to be enhanced advantageously at the transcription level by using strong transcription signals such as promoters and/or enhancers. However, in addition, it is also possible to enhance translation by, for example, improving the stability of the mRNA.

Other preferred sequences for use in plant gene expression cassettes are targeting-sequences necessary to direct the gene product in its

appropriate cell compartment (for review see Kermode, 1996 Crit. Rev. Plant Sci. 15(4):285-423 and references cited therein) such as the vacuole, the nucleus, all types of plastids like amyloplasts, chloroplasts, chromoplasts, the extracellular space, mitochondria, the endoplasmic reticulum, oil bodies, peroxisomes and other compartments of plant cells.

Plant gene expression can also be facilitated via an inducible promoter (for review see Gatz, 1997 Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:89-108). Chemically inducible promoters are especially suitable if gene expression is wanted to occur in a time specific manner.

Table 1 lists several examples of promoters that may be used to regulate transcription of the glutaredoxin or thioredoxin nucleic acid coding sequences.

Tab. 1: Examples of Tissue-specific and Stress inducible promoters in plants

Expression	Reference
Cor78- Cold, drought, salt, ABA, wounding-inducible	Ishitani, *et al.*, Plant Cell 9:1935-1949 (1997). Yamaguchi-Shinozaki and Shinozaki, Plant Cell 6:251-264 (1994).
Rci2A - Cold, dehydration-inducible	Capel *et al.*, Plant Physiol 115:569-576 (1997)
Rd22 - Drought, salt	Yamaguchi-Shinozaki and Shinozaki, Mol Gen Genet 238:17-25 (1993).
Cor15A - Cold, dehydration, ABA	Baker *et al.*, Plant Mol. Biol. 24:701-713 (1994).
GH3- Auxin inducible	Liu *et al.*, Plant Cell 6:645-657 (1994)
ARSK1-Root, salt inducible	Hwang and Goodman, Plant J 8:37-43 (1995).
PtxA - Root, salt inducible	GenBank accession X67427
SbHRGP3 - Root specific	Ahn *et al.*, Plant Cell 8:1477-1490 (1998).
KST1 - Guard cell specific	Plesch *et al.*, unpublished manuscript; Müller-Röber *et al*, EMBO J. 14:2409-2416 (1995).
KAT1 - Guard cell	Plesch *et al.*, Gene 249:83-89 (2000)

specific	Nakamura *et al.*, Plant Physiol. 109:371-374 (1995)
salicylic acid inducible	PCT Application No. WO 95/19443
tetracycline inducible	Gatz et al. Plant J. 2:397-404 (1992)
Ethanol inducible	PCT Application No. WO 93/21334
pathogen inducible PRP1	Ward et al., 1993 Plant. Mol. Biol. 22:361-366
heat inducible hsp80	U.S. Patent No. 5187267
cold inducible alpha-amylase	PCT Application No. WO 96/12814
Wound-inducible pinII	European Patent No. 375091
RD29A - salt-inducible	Yamaguchi-Shinozalei et al. (1993) Mol. Gen. Genet. 236:331-340
plastid-specific viral RNA-polymerase	PCT Application No. WO 95/16783 and. WO 97/06250

Other selection marker systems, like the AHAS marker or other promotors, e.g. superpromotor (Ni et al,., Plant Journal 7, 1995: 661-676), Ubiquitin promotor (Callis et al., J. Biol. Chem., 1990, 265: 12486-12493; US 5,510,474; US 6,020,190; Kawalleck et al., Plant. Molecular Biology, 1993, 21: 673-684) or 34S promotor (GenBank Accession numbers M59930 and X16673) were similar useful for the instant invention and are known to a person skilled in the art.

The invention further provides a recombinant expression vector comprising a glutaredoxin or thioredoxin DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner that allows for expression (by transcription of the DNA molecule) of an RNA molecule that is antisense to a glutaredoxin or thioredoxin mRNA. Regulatory sequences operatively linked to a nucleic acid molecule cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types. For

instance, viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus wherein antisense nucleic acids are produced under the control of a high efficiency regulatory region. The activity of the regulatory region can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, Reviews - Trends in Genetics, Vol. 1(1) 1986 and Mol et al., 1990 FEBS Letters 268:427-430.

Gene expression in plants is regulated by the interaction of protein transcription factors with specific nucleotide sequences within the regulatory region of a gene. A common type of transcription factor contains zinc finger (ZF) motifs. Each ZF module is approximately 30 amino acids long folded around a zinc ion. The DNA recognition domain of a ZF protein is a α-helical structure that inserts into the major grove of the DNA double helix. The module contains three amino acids that bind to the DNA with each amino acid contacting a single base pair in the target DNA sequence. ZF motifs are arranged in a modular repeating fashion to form a set of fingers that recognize a contiguous DNA sequence. For example, a three-fingered ZF motif will recognize 9 bp of DNA. Hundreds of proteins have been shown to contain ZF motifs with between 2 and 37 ZF modules in each protein (Isalan M, et al., 1998 Biochemistry 37(35):12026-33; Moore M, et al., 2001 Proc. Natl. Acad. Sci. USA 98(4):1432-1436 and 1437-1441; US patents US 6007988 and US 6013453).

The regulatory region of a plant gene contains many short DNA sequences (cis-acting elements) that serve as recognition domains for transcription factors, including ZF proteins. Similar recognition domains in

different genes allow the coordinate expression of several genes encoding enzymes in a metabolic pathway by common transcription factors. Variation in the recognition domains among members of a gene family facilitates differences in gene expression within the same gene family, for example, among tissues and stages of development and in response to environmental conditions.

Typical ZF proteins contain not only a DNA recognition domain but also a functional domain that enables the ZF protein to activate or repress transcription of a specific gene. Experimentally, an activation domain has been used to activate transcription of the target gene (US patent 5789538 and patent application WO9519431), but it is also possible to link a transcription repressor domain to the ZF and thereby inhibit transcription (patent applications WO00/47754 and WO2001002019). It has been reported that an enzymatic function such as nucleic acid cleavage can be linked to the ZF (patent application WO00/20622)

The invention provides a method that allows one skilled in the art to isolate the regulatory region of one or more Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin genes from the genome of a plant cell and to design zinc finger transcription factors linked to a functional domain that will interact with the regulatory region of the gene. The interaction of the zinc finger protein with the plant gene can be designed in such a manner as to alter expression of the gene and thereby confer increased or decreased tolerance of abiotic stress such as drought. The invention provides a method of producing a transgenic plant with a transgene encoding this designed transcription factor, or alternatively a natural transcription factor, that modifies transcription of the Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin gene to provide increased tolerance of environmental stress.

In particular, the invention provides a method of producing a transgenic plant with a Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin coding nucleic acid, wherein expression of the nucleic acid(s) in the plant results in increased tolerance to environmental stress as compared to a wild type plant comprising: (a) transforming a plant cell with an expression vector comprising a glutaredoxin or thioredoxin nucleic acid, and (b) generating from the plant cell a transgenic plant with an increased tolerance to environmental stress as compared to a wild type plant. For such plant transformation, binary vectors such as pBinAR can be used (Höfgen and Willmitzer, 1990 Plant Science 66:221-230). Moreover suitable binary vectors such as pBIN19, pBI101, pGPTV or pCambia are described in Hellens et al., Trends in Plant Science, 2000, 5: 446-451.

Construction of the binary vectors can be performed by ligation of the cDNA in sense or antisense orientation into the T-DNA. 5-prime to the cDNA a plant promoter activates transcription of the cDNA. A polyadenylation sequence is located 3-prime to the cDNA. Tissue-specific expression can be achieved by using a tissue specific promoter as listed above. Also, any other promoter element can be used. For constitutive expression within the whole plant, the CaMV 35S promoter can be used. The expressed protein can be targeted to a cellular compartment using a signal peptide, for example for plastids, mitochondria or endoplasmic reticulum (Kermode, 1996 Crit. Rev. Plant Sci. 4(15):285-423). The signal peptide is cloned 5-prime in frame to the cDNA to archive subcellular localization of the fusion protein. Additionally, promoters that are responsive to abiotic stresses can be used with, such as the *Arabidopsis* promoter RD29A. One skilled in the art will recognize that the promoter used should be operatively linked to the nucleic acid such that the promoter causes transcription of the nucleic acid which results in the synthesis of a mRNA which encodes a polypeptide.

Alternatively, the RNA can be an antisense RNA for use in affecting subsequent expression of the same or another gene or genes.

Alternate methods of transfection include the direct transfer of DNA into developing flowers via electroporation or *Agrobacterium* mediated gene transfer. *Agrobacterium* mediated plant transformation can be performed using for example the GV3101(pMP90) (Koncz and Schell, 1986 Mol. Gen. Genet. 204:383-396) or LBA4404 (Ooms et al., Plasmid, 1982, 7: 15-29; Hoekema et al., Nature, 1983, 303: 179-180) *Agrobacterium tumefaciens* strain. Transformation can be performed by standard transformation and regeneration techniques (Deblaere et al., 1994 Nucl. Acids. Res. 13:4777-4788; Gelvin and Schilperoort, Plant Molecular Biology Manual, 2nd Ed. - Dordrecht : Kluwer Academic Publ., 1995. - in Sect., Ringbuc Zentrale Signatur: BT11-P ISBN 0-7923-2731-4; Glick, B R and Thompson, J E, Methods in Plant Molecular Biology and Biotechnology, Boca Raton : CRC Press, 1993. - 360 S., ISBN 0-8493-5164-2). For example, rapeseed can be transformed via cotyledon or hypocotyl transformation (Moloney et al., 1989 Plant Cell Reports 8:238-242; De Block et al., 1989 Plant Physiol. 91:694-701). Use of antibiotics for *Agrobacterium* and plant selection depends on the binary vector and the *Agrobacterium* strain used for transformation. Rapeseed selection is normally performed using kanamycin as selectable plant marker. *Agrobacterium* mediated gene transfer to flax can be performed using, for example, a technique described by Mlynarova et al., 1994 Plant Cell Report 13:282-285. Additionally, transformation of soybean can be performed using for example a technique described in European Patent No. 0424 047, U.S. Patent No. 5,322,783, European Patent No. 0397 687, U.S. Patent No. 5,376,543 or U.S. Patent No. 5,169,770. Transformation of maize can be achieved by particle bombardment, polyethylene glycol mediated DNA uptake or via the silicon carbide fiber technique. (See, for example, Freeling

and Walbot "The maize handbook" Springer Verlag: New York (1993) ISBN 3-540-97826-7). A specific example of maize transformation is found in U.S. Patent No. 5,990,387 and a specific example of wheat transformation can be found in PCT Application No. WO 93/07256.

The Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin nucleic acid molecules of the invention have a variety of uses. Most importantly, the nucleic acid and amino acid sequences of the present invention can be used to transform plant cells or plants, thereby inducing tolerance to stresses such as drought, high salinity and cold. The present invention therefore provides a transgenic plant transformed by a Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin nucleic acid (coding or antisense), wherein expression of the nucleic acid sequence in the plant results in increased tolerance to environmental stress as compared to a wild type plant. The transgenic plant can be a monocot or a dicot or a gymnosperm plant. The invention further provides that the transgenic plant can be selected from maize, wheat, rye, oat, triticale, rice, barley, soybean, peanut, cotton, borage, sufflower, linseed, primrose, rapeseed, canola and turnip rape, manihot, pepper, sunflower, tagetes, solanaceous plant such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa, bushy plants such as coffee, cacao, tea, Salix species, trees such as oil palm, coconut, perennial grass, such as ryegrass and fescue, and forage crops, such as alfalfa and clover and Arabidopsis thaliana. Further the transgenic plant can be selected from spruce, pine or fir for example.

In particular, the present invention describes using the expression of Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin to engineer drought-tolerant, salt-tolerant and/or cold-tolerant plants. This strategy has herein been demonstrated for *Arabidopsis thaliana*,

Ryegrass, Alfalfa, Rapeseed/Canola, Soybean, Corn and Wheat but its application is not restricted to these plants. Accordingly, the invention provides a transgenic plant containing a glutaredoxin or thioredoxin selected from SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants, wherein the environmental stress is drought, increased salt or decreased or increased temperature but its application is not restricted to these adverse environments. Protection against other adverse conditions such as heat, air pollution, heavy metals and chemical toxicants, for example, may be obtained. In preferred embodiments, the environmental stress is drought.

The present invention also provides methods of modifying stress tolerance of a plant comprising, modifying the expression of a Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin in the plant. The invention provides that this method can be performed such that the stress tolerance is either increased or decreased. This can for example be done by the use of transcription factor or some type of site specific mutagenesis agent. In particular, the present invention provides methods of producing a transgenic plant having an increased tolerance to environmental stress as compared to a wild type plant comprising increasing expression of a Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin in a plant.

The Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin encoding nucleic acids of the present invention have utility as (Quantitative Trait Locus) QTL markers for mapping genetic loci associated with environmental stress tolerance. As such, the sequences have utility in the identification of plants that exhibit an environmental stress tolerance phenotype from those that do not within a segregating population of plants. For example, to identify the region of the genome to which a particular

glutaredoxin or thioredoxin nucleic acid sequence binds, genomic DNA could be digested with one or more restriction enzymes, and the fragments incubated with the glutaredoxin or thioredoxin nucleic acid, preferably with readily detectable labels. Binding of such a nucleic acid molecule to the genome fragment enables the localization of the fragment to the genome map and, when performed multiple times with different enzymes, facilitates a unique identifying pattern. Further, the nucleic acid molecules of the invention may be sufficiently homologous to the sequences of related species such that these nucleic acid molecules may serve as markers for the construction of a genomic map.

The genetics of quantitative traits associated to DNA markers has been used extensively in plant breeding for more than a decade (Tansgley et al., 1989 Biotechnology 7:257-264). The principle consists of using segregating lines derived from two homozygous parents and mapping these progeny with markers to link each marker to at least another one (saturated map), after which a statistical relationship between the quantitative trait value and the genotype at each marker is determined. A significant link of a locus to the trait means that at least one gene that in the vicinity of the marker contributes part of the phenotype variability. By definition, this locus is called a quantitative trait locus (QTL). In such a case, the gene becomes a candidate gene for explaining part of the observed phenotype and methods to identify and clone these genes have been described (Yano M, 2001. Current Opinion in Plant Biology 4:130-135). An observed correlation between a QTL and a gene location is likely to be causal, and therefore much more informative than a physiological correlation. This approach was applied to biochemical traits related to carbohydrate metabolism in maize leaves (Causse M., et al., 1995. Molecular Breeding 1:259-272).

This invention uses an alternative approach to the classical method. The approach of this invention is to use the QTL methodology linking a gene or locus known to be associated with the phenotype as a screening method. The marker may be associated with either the DNA sequences or the expression level of the gene, e.g. quantity of a specific mRNA molecule. In this instance, the marker serves as a convenient genetic means to identify individuals with the stress tolerance phenotype within a population of individuals that lack the phenotype. This method has utility when the phenotype is often difficult or expensive to detect or quantitative.

Many traits including tolerance of environmental stress and yield are associated with multiple genes and are therefore considered quantitative traits. This means that more than one marker or genetic locus is associated with the phenotype. In many instances, it is necessary to stack the various loci related to a phenotype. This is accomplished in standard plant breeding methods by cross-pollinating two parents with different loci (markers) contributing to the phenotype and selecting those progeny that have both markers. This process or breeding and selecting can be repeated multiple times to combine all loci into one progeny.

This invention provides markers of specific genetic loci that are associated with tolerance of abiotic environmental stress. The DNA sequences in SEQ IDs 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants may be used in the identification and selection of stress tolerant plants. These plants, their seeds and varieties derived from them would not contain transgenes but would contain alleles or genetic loci representing natural genetic diversity and thereby exhibit increased tolerance of abiotic environmental stress.

Growing the modified plant under less than suitable conditions and then analyzing the growth characteristics and/or metabolism can assess the

effect of the genetic modification in plants on stress tolerance. Such analysis techniques are well known to one skilled in the art, and include dry weight, wet weight, protein synthesis, carbohydrate synthesis, lipid synthesis, evapotranspiration rates, general plant and/or crop yield, flowering, reproduction, seed setting, root growth, respiration rates, photosynthesis rates, etc. (Applications of HPLC in Biochemistry in: Laboratory Techniques in Biochemistry and Molecular Biology, vol. 17; Rehm et al., 1993 Biotechnology, vol. 3, Chapter III: Product recovery and purification, page 469-714, VCH: Weinheim; Belter, P.A. et al., 1988 Bioseparations: downstream processing for biotechnology, John Wiley and Sons; Kennedy, J.F. and Cabral, J.M.S., 1992 Recovery processes for biological materials, John Wiley and Sons; Shaeiwitz, J.A. and Henry, J.D., 1988 Biochemical separations, in: Ulmann's Encyclopedia of Industrial Chemistry, vol. B3, Chapter 11, page 1-27, VCH: Weinheim; and Dechow, F.J. (1989) Separation and purification techniques in biotechnology, Noyes Publications).

The engineering of one or more Oxidoreductase Stress-Related Protein, particularly glutaredoxin or thioredoxin genes of the invention may also result in Oxidoreductase Stress- Related Protein, particularly glutaredoxin or thioredoxin proteins having altered activities which indirectly impact the stress response and/or stress tolerance of plants. For example, the normal biochemical processes of metabolism result in the production of a variety of products (e.g., hydrogen peroxide and other reactive oxygen species) which may actively interfere with these same metabolic processes (for example, peroxynitrite is known to react with tyrosine side chains, thereby inactivating some enzymes having tyrosine in the active site (Groves, J.T., 1999 Curr. Opin. Chem. Biol. 3(2):226-235). By optimizing the activity of one or more Oxidoreductase Stress-Related Protein, particularly glutaredoxin or

thioredoxin enzymes of the invention, it may be possible to improve the stress tolerance of the cell.

Additionally, the sequences disclosed herein, or fragments thereof, can be used to generate knockout mutations in the genomes of various plant cells (Girke, T., 1998 The Plant Journal 15:39-48). The resultant knockout cells can then be evaluated for their ability or capacity to tolerate various stress conditions, their response to various stress conditions, and the effect on the phenotype and/or genotype of the mutation. For other methods of gene inactivation see U.S. Patent No. 6004804 "Non-Chimeric Mutational Vectors" and Puttaraju et al., 1999 Spliceosome-mediated RNA *trans*-splicing as a tool for gene therapy Nature Biotechnology 17:246-252.

Throughout this application, various publications are referenced. The disclosures of all of these publications and those references cited within those publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It should also be understood that the foregoing relates to preferred embodiments of the present invention and that numerous changes may be made therein without departing from the scope of the invention. The invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof, which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

The invention also pertains the use of ORSRP encoding nucleic acid selected form the group comprising SEQ ID No. SEQ ID No. 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants and/or homologs thereof for preparing a plant cell with increased environmental stress tolerance. The said sequences can also be used for preparing a plant with increased environmental stress tolerance. Object of the invention is further the use of ORSRP encoding nucleic acid selected form the group comprising SEQ ID No. SEQ ID No. 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants and/or homologs thereof or parts thereof as DNA markers for selection of plants with increased tolerance to environmental stress. The said ORSRP encoding nucleic acid selected from the group comprising of SEQ ID No. SEQ ID No. 1, 3, 5, 7, 9, 11, 13 of yeast and/or SEQ ID No. 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49 of plants and/or homologs thereof or parts thereof can also be used as Quantitative Trait Locus (QTL) markers for mapping genetic loci associated with environmental stress tolerance.

Example 1

Engineering stress-tolerant Arabidopsis plants by over-expressing glutaredoxin or thioredoxin genes.

Gene cloning and Transformation of Arabidopsis thaliana

Amplification The standard protocol of *Pfu* DNA polymerase or a *Pfu/Taq* DNA polymerase mix was used for the amplification procedure. Amplified ORF fragments were analysed by gel electrophoresis. Each primer consists of a universal 5 `end and ORF specific 3` end whereby the universal sequences differ for the forward and reverse primers (forward primer sequence contains a *Eco*RI and the reverse primer sequence a *Sma*I restriction site) allowing a unidirectional cloning success.

Amplification using the protocol of *Pfu* or Herculase DNA polymerase (Stratagene). Conditions: 1x PCR buffer [20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10mM $(NH_4)SO_4$, 0.1 % Triton X-100, 0.1 mg/ml BSA], 100 ng genomic DNA *Saccharomyces cerevisae* (S288C), 50 pmol forward primer, 50 pmol reverse primer, 2.5 u *Pfu* or Herculase DNA polymerase. 1st cycle for 3` at 94 °C, followed by 25 cycles for 30`` at 94°C, 30`` 55 °C and 5-6` 72 °C, followed by 1 cycle for 6-10` at 72 °C, final for 4 °C at ∞.

YDR513w primer forward:
GGAATTCCAGCTGACCACCATGGAGACCAATTTTTCCTTCGACT

YDR513w primer reverse:
GATCCCCGGGAATTGCCATGCTATTGAAATACCGGCTTCAATATTT

YER174c primer forward:
GGAATTCCAGCTGACCACCATGACTGTGGTTGAAATAAAAAGCC

YER174c primer reverse:
GATCCCCGGGAATTGCCATGTTACTGTAGAGCATGTTGGAAATATT

Vector preparation. The preferred binary vector 1bxbigResgen, which is based on the modified pPZP binary vector backbone (comprising the kanamycin-gene for bacterial selection; Hajukiewicz, P. et al., 1994, plant Mol. Biol., 25: 989-994) carried the selection marker bar-gene (De Block et al., 1987, EMBO J. 6, 2513-2518) driven by the mas1´promotor (Velten et al., 1984, EMBO J. 3, 2723-2730; Mengiste, Amedeo and Paszkowski, 1997, Plant J., 12, 945-948) on its T-DNA. In addition the T-DNA contained the strong double 35S promotor (Kay et al., 1987, Science 236, 1299-1302) in front of a cloning cassette followed by the nos-terminator (Depicker A. Stachel S. Dhaese P. Zambryski P. Goodman HM. Nopaline synthase: transcript mapping and DNA sequence. Journal of Molecular & Applied Genetics. 1(6):561-73, 1982.). The cloning cassette consists of the following sequence:

5`-GGAATTCCAGCTGACCACCATGGCAATTCCCGGGGATC-3´

Other selection marker systems, like the AHAS marker or other promotors, e.g. superpromotor (Ni-Min et al,., Plant Journal, 1995, 7(4): 661-676), Ubiquitin promotor (Callis et al., J. Biol. Chem., 1990, 265: 12486-12493; US 5,510,474; US 6,020,190; Kawalleck et al., Plant. Molecular Biology, 1993, 21: 673-684) or 34S promotor (GenBank Accession numbers M59930 and X16673) were similar useful for the instant invention and are

known to a person skilled in the art. The vector was linearised with *EcoRI* and *SmaI* using the standard protocol provided by the supplier (MBI Fermentas, Germany) and purified using Qiagen columns (Qiagen, Hilden, Germany).

Ligation and transformation Present ORF fragments (~ 100 ng) were digested by *Eco*RI and *Sma*I using the standard protocol provided by the supplier (MBI Fermentas, Germany), purified using Qiagen columns (Qiagen, Hilden, Germany) and were ligated into the cloning cassette of the binary vector systems (~ 30 ng) using standard procedures (Maniatis et al.).

Ligation products were transformed into *E.coli* (DH5alpha) using a standard heat shock protocol (Maniatis et al.). Transformed colonies were grown on LB media and selected by respective antibiotica (Km) for 16h at 37 °C ÜN.

Plasmidpreparation Plasmids were prepared using standard protocol (Qiagen Hilden, Germany).

Transformation of Agrobacteria Plasmids were transformed into *Agrobacterium tumefaciens* (GV3101pMP90; Koncz and Schell, 1986 Mol. Gen. Genet. 204:383-396) using heat shock or electroporation protocols. Transformed colonies were grown on YEP media and selected by respective antibiotika (Rif/Gent/Km) for 2d at 28 °C ÜN. These agrobacteria cultures were used for the plant transformation.

Arabidopsis thaliana was grown and transformed according to standard conditions (Bechtold 1993 (Bechtold, N., Ellis, J., Pelletier, G. 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of *Arabidopsis thaliana* plantsC.R. Acad.Sci.Paris. 316:1194-1199); Bent et al. 1994 (Bent, A., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung,

J., and Staskawicz, B.J. 1994; PPCS2 of *Arabidopsis thaliana*: A leucin-rich repeat class of plant disease resistant genes; Science 265:1856-1860).

Transgenic *A. thaliana* plants were grown individually in pots containing a 4:1 (v/v) mixture of soil and quartz sand in a York growth chamber. Standard growth conditions were: photoperiod of 16 h light and 8 h dark, 20°C, 60% relative humidity, and a photon flux density of 150 µE. To induce germination, sown seeds were kept at 4°C, in the dark, for 3 days. Plants were watered daily until they were approximately 3 weeks old at which time drought was imposed by withholding water. Coincidentally, the relative humidity was reduced in 10% increments every second day to 20%. After approximately 12 days of withholding water, most plants showed visual symptoms of injury, such as wilting and leaf browning, whereas tolerant plants were identified as being visually turgid and healthy green in color. Plants were scored for symptoms of drought injury in comparison to neighbouring plants for 3 days in succession.

Three successive experiments were conducted. In the first experiment, 10 independent T2 lines were sown for each gene being tested. The percentage of plants not showing visual symptoms of injury was determined. In the second experiment, the lines that had been scored as tolerant in the first experiment were put through a confirmation screen according to the same experimental procedures. In this experiment, 10 plants of each tolerant line were grown and treated as before. In the third experiment, at least 5 replicates of the most tolerant line were grown and treated as before. The average and maximum number of days of drought survival after wild-type control had visually died and the percentage tolerant plants was determined. Additionally measurements of chlorophyll fluorescence were made in stressed and non-stressed plants using a Mini-PAM (Heinz Walz GmbH, Effeltrich, Germany).

In the first experiment, after 12 days of drought, the control, non-transgenic *Arabidopsis thaliana* and most transgenic lines expressing other transgenes in the test showed extreme visual symptoms of stress including necrosis and cell death. Several plants expressing the YER174C (=ORF737; SEQ ID No. 7) gene and the YDR513W (=ORF809; SEQ ID No. 3) gene retained viability as shown by their turgid appearance and maintenance of green color. Several independent transgenic lines, in the case of both the YER174C and the YDR513W genes, did not become necrotic for at least 3 days after the control plants had died (Table 2 and 3).

The second experiment compared a smaller number of independent transgenic lines for each gene but a greater number of progeny within each independent transformation event. This experiment confirmed the previous results. Those lines containing the YER174C gene (Table 2) did not become necrotic for 1-2 days after the controls and in the case of the YDR513W gene, 2-3 days after the controls (Table 3).

Table 2: Drought tolerance of transgenic *Arabidopsis thaliana* expressing the YER174C gene after imposition of drought stress on 3 week old plants. Control plants showed extensive visual symptoms of injury on day 12 and were considered dead.

		Percent survival		
Experiment	Plant	Day 13	Day 14	Day 15
1	Control	0	0	0
	Transgenic 737	60	40	20
2	Control	0	0	0
	Transgenic 737-	22	22	0

1			
Transgenic 737-3	50	0	0

Table 3: Drought tolerance of transgenic *Arabidopsis thaliana* expressing the YDR513W gene after imposition of drought stress on 3 week old plants. Control plants showed extensive visual symptoms of injury on day 12 and were considered dead.

		Percent survival		
Experiment	Plant	Day 13	Day 14	Day 15
1	Control	0	0	0
	Transgenic 809	50	33	33
2	Control	0	0	0
	Transgenic 809-5	25	13	13
	Transgenic 809-8	50	25	0

In the third experiment, one transgenic line from each gene was tested using a even larger number of plants. In line 737-3 expressing the YER174C gene, necrosis did not occur on average until 1.1 days after the controls and 2 of the 22 plants tested did not show necrosis until 4 days later (Table 4). Similarly, line 809-8 expressing the YDR513W gene survived on average 3.1 days longer than the control and 1 plant survived for 6 days longer later

(Table 4). Other independent transgenic lines for both genes showed greater survival than the non-transgenic plants in this experiment.

Chlorophyll fluorescence measurements of photosynthetic yield confirmed that 12 days of drought stress completely inhibited photosynthesis in the control plants, but the transgenic line 809-8 maintained its photosynthetic function longer (Table 5).

Table 4: Relative drought tolerance of *Arabidopsis thaliana* transgenic line 737-3 expressing the YER174C gene and line 809-8 expressing the YDR513W gene after imposition of drought stress on 3 week old plants in comparison to non-transgenic control plants. Control plants showed extensive visual symptoms of injury on day 12 and were considered dead.

	737-3	809-8
Number of plants tested	22	7
Duration of survival after control (days)	1.1	3.1
Maximal duration of survival (number of plants)	3 (2)	6 (1)

Table 5: Effect of drought stress on photosynthetic yield as determined by chlorophyll fluorescence (± std deviation) of *Arabidopsis thaliana* control and transgenic line 809-8 expressing the YDR513W gene.

Days of drought	Control	Transgenic line 809-8
0	765 ± 29	723 ± 29
5	794 ± 36	781 ± 25
10	412 ± 194	660 ± 121
12	54 ± 83	411 ± 305

Example 2

Isolation and Characterization of plant glutaredoxin genes

ORF 737 and 809 correspond to yeast, *Saccharomyces cerevisiae*, genes for glutaredoxin4 (GRX4) and glutaredoxin2 (GRX2), respectively, that contain a pair of cysteine amino acids at the putative active site of the protein (Grant CM. 2000. Molecular Microbiology 39: 533-541; Grant CM et al., 2001. Biochimica et Biophysica Acta - Gene Structure & Expression 1490: 33-42). Grx3, Grx4, and Grx5 is a subfamily of yeast glutaredoxins that contain a single cysteine residue at the putative active site (Rodriguez-Manzaneque et al., 1999. Molecular & Cellular Biology 19: 8180-8190). *Saccharomyces cerevisiae* also contains two gene pairs for thioredoxins (TRX1, TRX2) (Draculic et al., 2000. Molecular Microbiology 36: 1167-1174). These gene sequences are listed in GenBank under the accession numbers listed in Table 6.

The sequence of GRX2 and GRX4 was used to identify related gene sequences in *Arabidopsis thaliana* by Blast analysis (Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 J Mol Biol 215(3):403-10). The results identified related sequences with $E< 10^{-10}$ as shown in Table 6, where E is defined as the expectancy value, or the statistical probability that the sequence appears in the database at random.. A similar analysis was done on a three libraries of expressed sequence tags (ESTs) from *Brassica napus* cv. "AC Excel", "Quantum" and "Cresor" (canola) and *Oryza sativa* cv. Nippon-Barre (a japonica rice). The search identified several Brassica and rice glutaredoxin cDNA sequences with $E< 10^{-10}$ (Table 6).

The yeast and plant cDNA sequences were translated into a predicted amino acid sequences and the relationship among the amino acid sequences was determined by sequence alignment and block alignment using the ClustalW algorithm in Vector NTI ver7. The glutaredoxin and thioredoxin genes were separated into four subfamilies based on this alignment as shown in Figure 1. The glutaredoxin family is characterized by the standard glutaredoxin domain defined in the Prosite database as an amino acid motif with the consensus sequence [LIVMD]-[FYSA]-x(4)-C-[PV]-[FYWH]-C-x(2)-[TAV]-x(2,3)- [LIV]. Most sequences show the characteristic two cysteines that when reduced form either two thiol groups or when oxidized form a disulfide bond. Other proteins in this family have only a single C at this site.

Subfamily 1 contains the yeast genes GRX1 and GRX2 (figures 2-4). Domain 1 has the core sequence [VI]-[VF]-[VI]-X-[SA]-K-[TS]-[WY]-C-[PGS]-[YF]-[CS]. OZ1116C26232 and AtQ95K75 lack the C-X-X-C disulfide site and instead have a single C at this site. Domain 2 contains a motif defined as G-Q-X-T-V-P-N-[VI]-[FY]-[VI]-X-G-[KN]-H-I-G-G-[CN].

Subfamily 2 contains both glutaredoxin GRX3 and GRX4 and thioredoxin THX1 and THX2 sequences (figures 5-7). This family has a region of homology comprising two domains. In most sequences the domains are continuous, except in GRX3 and GRX4 in which the two domains are separated by two amino acids. Domain 1 has a core sequence of [VI]-V-[VL]-X-F-X-[TA]-X-W-[CA]-X-[PA]-[CS]-K. The region [CA]-X(2)-[CS] contains C at position 1 or 4 or both. Domain 2 is a region of similarity that has a core sequence of F-X(2)-[VI]-[ED]-[AV]-[ED]-E-X(2)-[ED]-[IV].

Subfamily 3 contains GRX5 and three plant sequences that have a single C amino acid at the putative active site (figures 8-11). The core sequence of domain 1 is V-[VM]-X(3)-K-G-X(4)-P-X-C-G-F-S. Domain 2 is defined by the sequence Q-[LI]-[FY]-[VI]-X-[GK]-E-[FL]-X-G-G-[CS]-D-[IV].

Subfamily 4 does not have any members from yeast and is comprised of 5 plant sequences that have two domains of homology (figures 11-13). Domain 1 has a core sequence similar to subfamily 1 that is [VI]-V-I-F-S-K-S-Y-C-P-Y-C. Domain 2 has two regions with common sequences of V-V-E-L-D-X-R-E-D-G and V-G-R-R-T-V-P-Q-V-F-[VI]-[NH]-G-K-H-[LI]-G-G-S-D-D.

A representative of each subfamily was selected and the full length coding sequence was ligated into a plant transformation vector using standard molecular biology techniques as described in Example 1. The coding sequence was inserted at the 3' end of a constitutive promoter to control expression in plants. The vector was transferred to *Agrobacterium tumefaciens* and this strain was used to transform *Arabidopsis thaliana* as described in Example 1. Transgenic plants were grown and treated with drought stress as described in Example 1. Those plants that contained the glutaredoxin/thioredoxin transgene from subfamilies 1, 2 and 3 were more tolerant of the drought treatment than the control, non-transgenic plants.

Table 6. Summary of yeast and plant glutaredoxin coding sequences. Query specifies the ORF sequence used for the Blast search

source	Sub Family	query	Gene ID	GenBank Accession	Nucleotide SEQ ID No.	Amino Acid SEQ ID No.
Yeast	1		GRX1	X59720	1	2
	1	809	GRX2	U18922	3	4
	2		GRX3	Z47746	5	6
	2	737	GRX4	U33057	7	8
	3		GRX5	U39205	9	10
	2		THX1	M59168	11	12
	2		THX2	M59169	13	14
Brassica	1	809	BN1106 C12219	NA	15	16
	4	809	BN1106 C21909	NA	17	18
	1	809	BN1106 C2202	NA	19	20
	4	809	BN1106 C2582	NA	21	22
	2	737	BN1106 C23043	NA	23	24
Arabi-dopsis	1	809	AtQ9FM49	AB009051	25	26
	1	809	AtQ9FNE2	AB006702	27	28
	4	809	AtQ9FVX1	NM_106386	29	30
	4	809	AtQ9M457	ATH271472	31	32
	1	809	AtQ9SK75	AY094445	33	34
	3	737	AtQ9LW13	AY087154	35	36
	3	737	AtQ9SV38	AY078020	37	38
	3	737	AtO80451	AY086273	39	40
	2	737	AtO65541	NM_119410	41	42
	2	737	AtQ9ZPH2	AY058202	43	44
Rice	4	809	OZ1116 C12744	NA	45	46
	1	809	OZ1116 C2194	X77150	47	48
	1	809	OZ1116 C26232	NA	49	50

NA- not available; sequence is not in a GenBank database

Example 3

Engineering stress-tolerant Arabidopsis plants by over-expressing glutaredoxin or thioredoxin genes using stress-inducible and tissue-specific promoters.

Transgenic Arabidopsis plants were created as in example 1 to express the glutaredoxin and thioredoxin transgenes under the control of either a tissue-specific or stress-inducible promoter. Constitutive expression of a transgene may cause deleterious side effects. Stress inducible expression was achieved using promoters selected from those listed above in Table 1.

T2 generation plants were produced and treated with drought stress in two experiments. For the first drought experiment, the plants were deprived of water until the plant and soil were desiccated. At various times after withholding water, a normal watering schedule was resumed and the plants were grown to maturity. Seed yield was determined as g seeds per plant. At an equivalent degree of drought stress, tolerant plants were able to resume normal growth and produced more seeds than non-transgenic control plants. Proline content of the leaves and stomatal aperture were also measured at various times during the drought stress. Tolerant plants maintained a lower proline content and a greater stomatal aperture than the non-transgenic control plants.

An alternative method to impose water stress on the transgenic plants was by treatment with water containing an osmolyte such as polyethylene glycol (PEG) at specific water potential. Since PEG may be toxic, the plants were given only a short term exposure and then normal watering was

resumed. As above, seed yields were measured from the mature plants. The response was measured during the stress period by physical measurements, such as stomatal aperture or osmotic potential, or biochemical measurements, such as accumulation of proline. Tolerant plants had higher seed yields, maintained their stomatal aperture and showed only slight changes in osmotic potential and proline levels, whereas the susceptible non-transgenic control plants closed their stomata and exhibited increased osmotic potential and proline levels.

The transgenic plants with a constitutive promoter controlling transcription of the transgene were compared to those plants with a drought-inducible promoter in the absence of stress. The results indicated that the metabolite and gene expression changes noted in examples 2 and 3 did not occur when plants with the stress-inducible promoter were grown in the absence of stress. These plants also had higher seed yields than those with the constitutive promoter.

Example 4

Inheritance and segregation of drought tolerance with the glutaredoxin and thioredoxin transgenes.

Transgenic Arabidopsis plants in the T2 generation were analyzed by PCR to confirm the presence of T-DNA. These results were confirmed by Southern hybridization in which DNA is electrophoresed on a 1% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics). The PCR DIG Probe Synthesis Kit (Roche Diagnostics) is used to prepare a digoxigenin-labelled probe by PCR, and used as recommended by the

manufacturer. Homozygous lines with single insertions of T-DNA were selected for cross-pollination experiments.

A homozygous line with the glutaredoxin transgene (GG) was cross-pollinated with a homozygous line with the thioredoxin transgene (TT). Since the transgenes are not at the same locus, the F1 progeny were heterozygous (G-T-). The F2 progeny segregated in a 9: 3: 3: 1 ratio of double transformants containing both transgenes, to single transformants containing either G or T, and nulls containing neither transgene. The genotype of the progeny was determined by PCR analysis for each of the transgenes. Homozygous lines of each genotype GGTT, GG--, --TT, and —— were identified by quantitative PCR and confirmed by inheritance patterns of the transgenes.

Homozygous lines were subjected to drought stress, metabolite analysis and expression profiling as described in examples 1, 2, 3 and 4. The transgenic lines were more drought tolerant than the null line, had altered metabolite levels consistent with the observations in example 2 and altered gene expression patterns consistent with the observations in example 3.

Example 5

Over-expression of glutaredoxin or thioredoxin genes provides tolerance of multiple abiotic stresses.

Plants that exhibit tolerance of one abiotic stress often exhibit tolerance of another environmental stress or an oxygen free radical generating herbicide. This phenomenon of cross-tolerance is not understood at a mechanistic level (McKersie and Leshem, 1994). Nonetheless, it is reasonable to expect that plants exhibiting enhanced drought tolerance due to the expression of a transgene might also exhibit tolerance of low temperatures, freezing, salt, air

pollutants such as ozone, and other abiotic stresses. In support of this hypothesis, the expression of several genes are up or down-regulated by mulitple abiotic stress factors including cold, salt, osmoticum, ABA, etc (e.g. Hong et al. (1992) Developmental and organ-specific expression of an ABA- and stress-induced protein in barley. Plant Mol Biol 18: 663-674; Jagendorf and Takabe (2001) Inducers of glycinebetaine synthesis in barley. Plant Physiol 127: 1827-1835); Mizoguchi et al. (1996) A gene encoding a mitogen-activated protein kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in Arabidopsis thaliana. Proc Natl Acad Sci U S A 93: 765-769; Zhu (2001) Cell signaling under salt, water and cold stresses. Curr Opin Plant Biol 4: 401-406).

To determine salt tolerance, seeds of *Arabidopsis thaliana* were sterilized (100% bleach, 0.1% TritonX for five minutes two times and rinsed five times with ddH2O). Seeds were plated on non-selection media (1/2 MS, 0.6% phytagar, 0.5g/L MES, 1% sucrose, 2 µg/ml benamyl). Seeds are allowed to germinate for approximately ten days. At the 4-5 leaf stage, transgenic plants were potted into 5.5cm diameter pots and allowed to grow (22°C, continuous light) for approximately seven days, watering as needed. To begin the assay, two liters of 100 mM NaCl and 1/8 MS was added to the tray under the pots. To the tray containing the control plants, three liters of 1/8 MS was added. The concentrations of NaCl supplementation were increased stepwise by 50 mM every 4 days up to 200 mM. After the salt treatment with 200 mM, fresh and dry weights of the plants as well as seed yields were determined.

To determine cold tolerance, seeds of the transgenic and cold lines were germinated and grown for approximately 10 days to the 4-5 leaf stage as above. The plants were then transferred to cold temperatures (5°C) and grown through the flowering and seed set stages of development.

Photosynthesis was measured using chlorophyll fluorescence as an indicator of photosynthetic fitness and integrity of the photosystems. Seed yield and plant dry weight were measured as an indictor of plant biomass production.

Plants that had tolerance to salinity or cold had higher seed yields, photosynthesis and dry matter production than susceptible plants.

Example 6

Engineering stress-tolerant alfalfa plants by over-expressing glutaredoxin or thioredoxin genes.

A regenerating clone of alfalfa *(Medicago sativa)* was transformed using the method of (McKersie et al., 1999 Plant Physiol 119: 839–847). Regeneration and transformation of alfalfa is genotype dependent and therefore a regenerating plant is required. Methods to obtain regenerating plants have been described. For example, these can be selected from the cultivar Rangelander (Agriculture Canada) or any other commercial alfalfa variety as described by Brown DCW and A Atanassov (1985. Plant Cell Tissue Organ Culture 4: 111-112). Alternatively, the RA3 variety (University of Wisconsin) has been selected for use in tissue culture (Walker et al., 1978 Am J Bot 65:654-659).

Petiole explants were cocultivated with an overnight culture of *Agrobacterium tumefaciens* C58C1 pMP90 (McKersie et al., 1999 Plant Physiol 119: 839–847) or LBA4404 containing a binary vector. Many different binary vector systems have been described for plant transformation (e.g. An, G. in Agrobacterium Protocols. Methods in Molecular Biology vol 44, pp 47-62, Gartland KMA and MR Davey eds. Humana Press, Totowa, New Jersey). Many are based on the vector pBIN19 described by Bevan (Nucleic Acid Research. 1984. 12:8711-8721) that includes a plant gene expression

cassette flanked by the left and right border sequences from the Ti plasmid of *Agrobacterium tumefaciens.* A plant gene expression cassette consists of at least two genes – a selection marker gene and a plant promoter regulating the transcription of the cDNA or genomic DNA of the trait gene. Various selection marker genes can be used including the Arabidopsis gene encoding a mutated acetohydroxy acid synthase (AHAS) enzyme (US patents 57673666 and 6225105). Similarly, various promoters can be used to regulate the trait gene that provides constitutive, developmental, tissue or environmental regulation of gene transcription. In this example, the 34S promoter (GenBank Accession numbers M59930 and X16673) was used to provide constitutive expression of the trait gene.

The explants were cocultivated for 3 d in the dark on SH induction medium containing 288 mg/ L Pro, 53 mg/ L thioproline, 4.35 g/ L K_2SO_4, and 100 μm acetosyringinone. The explants were washed in half-strength Murashige-Skoog medium (Murashige and Skoog, 1962) and plated on the same SH induction medium without acetosyringinone but with a suitable selection agent and suitable antibiotic to inhibit Agrobacterium growth. After several weeks, somatic embryos were transferred to BOi2Y development medium containing no growth regulators, no antibiotics, and 50 g/ L sucrose. Somatic embryos were subsequently germinated on half-strength Murashige-Skoog medium. Rooted seedlings were transplanted into pots and grown in a greenhouse.

The T0 transgenic plants were propagated by node cuttings and rooted in Turface growth medium. The plants were defoliated and grown to a height of about 10 cm (approximately 2 weeks after defoliation). The plants were then subjected to drought stress in two experiments.

For the first drought experiment, the seedlings received no water for a period up to 3 weeks at which time the plant and soil were desiccated. At various times after withholding water, a normal watering schedule was

resumed. At one week after resuming watering, the fresh and dry weights of the shoots was determined. At an equivalent degree of drought stress, tolerant plants were able to resume normal growth whereas susceptible plants had died or suffered significant injury resulting in less dry matter. Proline content of the leaves and stomatal aperture were also measured at various times during the drought stress. Tolerant plants maintained a lower proline content and a greater stomatal aperture than the non-transgenic control plants.

An alternative method to impose water stress on the transgenic plants was by treatment with a solution at specific water potential, containing an osmolyte such as polyethylene glycol (PEG). The PEG treatment was given to either detached leaves (e.g. Djilianov et al., 1997 Plant Science 129: 147-156) or to the roots (Wakabayashi et al., 1997 Plant Physiol 113: 967-973). Since PEG may be toxic, the plants were given only a short term exposure. The response was measured as physical measurements such as stomatal aperture or osmotic potential, or biochemical measurements such as accumulation of proline. Tolerant plants maintained their stomatal aperture and showed only slight changes in osmotic potential, whereas the susceptible non-transgenic control plants closed their stomata and exhibited increased osmotic potential. In addition the changes in proline and other metabolites were less in the tolerant transgenic plants than in the non-transgenic control plants.

Tolerance of salinity and cold were measured using methods as described in example 5. Plants that had tolerance to salinity or cold had higher seed yields, photosynthesis and dry matter production than susceptible plants.

Example 7

Engineering stress-tolerant ryegrass plants by over-expressing glutaredoxin or thioredoxin genes.

Seeds of several different ryegrass varieties may be used as explant sources for transformation, including the commercial variety Gunne available from Svalof Weibull seed company or the variety Affinity. Seeds were surface-sterilized sequentially with 1% Tween-20 for 1 minute, 100 % bleach for 60 minutes, 3 rinses with 5 minutes each with de-ionized and distilled H_2O, and then germinated for 3-4 days on moist, sterile filter paper in the dark. Seedlings were further sterilized for 1 minute with 1% Tween-20, 5 minutes with 75% bleach, and rinsed 3 times with ddH_2O, 5 min each.

Surface-sterilized seeds were placed on the callus induction medium containing Murashige and Skoog basal salts and vitamins, 20 g/l sucrose, 150 mg/l asparagine, 500 mg/l casein hydrolysate, 3 g/l Phytagel, 10 mg/l BAP, and 5 mg/l dicamba. Plates were incubated in the dark at 25C for 4 weeks for seed germination and embryogenic callus induction

After 4 weeks on the callus induction medium, the shoots and roots of the seedlings were trimmed away, the callus was transferred to fresh media, maintained in culture for another 4 weeks, and then transferred to MSO medium in light for 2 weeks. Several pieces of callus (11-17 weeks old) were either strained through a 10 mesh sieve and put onto callus induction medium, or cultured in 100 ml of liquid ryegrass callus induction media (same medium as for callus induction with agar) in a 250 ml flask. The flask was wrapped in foil and shaken at 175 rpm in the dark at 23 C for 1 week. Sieving the liquid culture with a 40-mesh sieve collected the cells. The fraction collected on the sieve was plated and cultured on solid ryegrass callus induction medium for 1 week in the dark at 25C. The callus was then

transferred to and cultured on MS medium containing 1% sucrose for 2 weeks.

Transformation can be accomplished with either Agrobacterium of with particle bombardment methods. An expression vector is created containing a constitutive plant promoter and the cDNA of the gene in a pUC vector. The plasmid DNA was prepared from *E. coli* cells using with Qiagen kit according to manufacturer's instruction. Approximately 2 g of embryogenic callus was spread in the center of a sterile filter paper in a Petri dish. An aliquot of liquid MSO with 10 g/l sucrose was added to the filter paper. Gold particles (1.0 µm in size) were coated with plasmid DNA according to method of Sanford et al., 1993 and delivered to the embryogenic callus with the following parameters: 500 µg particles and 2 µg DNA per shot, 1300 psi and a target distance of 8.5 cm from stopping plate to plate of callus and 1 shot per plate of callus.

After the bombardment, calli were transferred back to the fresh callus development medium and maintained in the dark at room temperature for a 1-week period. The callus was then transferred to growth conditions in the light at 25C to initiate embryo differentiation with the appropriate selection agent, e.g. 250 nM Arsenal, 5 mg/l PPT or 50 mg/L kanamycin. Shoots resistant to the selection agent appeared and once rotted were transferred to soil.
Samples of the primary transgenic plants (T0) are analyzed by PCR to confirm the presence of T-DNA. These results are confirmed by Southern hybridization in which DNA is electrophoresed on a 1% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics). The PCR DIG Probe Synthesis Kit (Roche Diagnostics) is used to prepare a digoxigenin-labelled probe by PCR, and used as recommended by the manufacturer.

Transgenic T0 ryegrass plants were propagated vegetatively by excising tillers. The transplanted tillers were maintained in the greenhouse for

2 months until well established. The shoots were defoliated and allowed to grow for 2 weeks.

The first drought experiment was conducted in a manner similar to that described in example 5. The seedlings received no water for a period up to 3 weeks at which time the plant and soil were desiccated. At various times after withholding water, a normal watering schedule was resumed. At one week after resuming watering, the lengths of leaf blades, and the fresh and dry weights of the shoots was determined. At an equivalent degree of drought stress, tolerant plants were able to resume normal growth whereas susceptible plants had died or suffered significant injury resulting in shorter leaves and less dry matter. Proline content of the leaves and stomatal aperture were also measured at various times during the drought stress. Tolerant plants maintained a lower proline content and a greater stomatal aperture than the non-transgenic control plants.

A second experiment imposing drought stress on the transgenic plants was by treatment with a solution of PEG as described in the previous examples. Tolerance of salinity and cold were measured using methods as described in example 5. Plants that had tolerance to salinity or cold had higher seed yields, photosynthesis and dry matter production than susceptible plants.

Example 8

Engineering stress-tolerant soybean plants by over-expressing glutaredoxin or thioredoxin genes.

Soybean was transformed according to the following modification of the method described in the Texas A&M patent US 5,164,310. Several commercial soybean varieties are amenable to transformation by this method. The cultivar Jack (available from the Illinois Seed Foundation) is a commonly

used for transformation. Seeds were sterilized by immersion in 70% (v/v) ethanol for 6 min and in 25 % commercial bleach (NaOCl) supplemented with 0.1% (v/v) Tween for 20 min, followed by rinsing 4 times with sterile double distilled water. Seven-day seedlings were propagated by removing the radicle, hypocotyl and one cotyledon from each seedling. Then, the epicotyl with one cotyledon was transferred to fresh germination media in petri dishes and incubated at 25°C under a 16-hr photoperiod (approx. 100 $\mu E\text{-}m^{-2}s^{-1}$) for three weeks. Axillary nodes (approx. 4 mm in length) were cut from 3 – 4 week-old plants. Axillary nodes were excised and incubated in *Agrobacterium* LBA4404 culture.

Many different binary vector systems have been described for plant transformation (e.g. An, G. in Agrobacterium Protocols. Methods in Molecular Biology vol 44, pp 47-62, Gartland KMA and MR Davey eds. Humana Press, Totowa, New Jersey). Many are based on the vector pBIN19 described by Bevan (Nucleic Acid Research. 1984. 12:8711-8721) that includes a plant gene expression cassette flanked by the left and right border sequences from the Ti plasmid of *Agrobacterium tumefaciens.* A plant gene expression cassette consists of at least two genes – a selection marker gene and a plant promoter regulating the transcription of the cDNA or genomic DNA of the trait gene. Various selection marker genes can be used including the Arabidopsis gene encoding a mutated acetohydroxy acid synthase (AHAS) enzyme (US patents 57673666 and 6225105). Similarly, various promoters can be used to regulate the trait gene to provide constitutive, developmental, tissue or environmental regulation of gene transcription. In this example, the 34S promoter (GenBank Accession numbers M59930 and X16673) was used to provide constitutive expression of the trait gene.

After the co-cultivation treatment, the explants were washed and transferred to selection media supplemented with 500 mg/L timentin. Shoots

were excised and placed on a shoot elongation medium. Shoots longer than 1 cm were placed on rooting medium for two to four weeks prior to transplanting to soil.

The primary transgenic plants (T0) were analyzed by PCR to confirm the presence of T-DNA. These results were confirmed by Southern hybridization in which DNA is electrophoresed on a 1% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics). The PCR DIG Probe Synthesis Kit (Roche Diagnostics) is used to prepare a digoxigenin-labelled probe by PCR, and used as recommended by the manufacturer.

Tolerant plants had higher seed yields, maintained their stomatal aperture and showed only slight changes in osmotic potential and proline levels, whereas the susceptible non-transgenic control plants closed their stomata and exhibited increased osmotic potential and proline levels.

Tolerance of salinity and cold were measured using methods as described in example 5. Plants that had tolerance to salinity or cold had higher seed yields, photosynthesis and dry matter production than susceptible plants.

Example 9

Engineering stress-tolerant Rapeseed/Canola plants by over-expressing glutaredoxin or thioredoxin genes.

Cotyledonary petioles and hypocotyls of 5-6 day-old young seedlings were used as explants for tissue culture and transformed according to Babic et al.(1998, Plant Cell Rep 17: 183-188). The commercial cultivar Westar (Agriculture Canada) is the standard variety used for transformation, but other varieties can be used.

Agrobacterium tumefaciens LBA4404 containing a binary vector was used for canola transformation. Many different binary vector systems have been described for plant transformation (e.g. An, G. in Agrobacterium Protocols. Methods in Molecular Biology vol 44, pp 47-62, Gartland KMA and MR Davey eds. Humana Press, Totowa, New Jersey). Many are based on the vector pBIN19 described by Bevan (Nucleic Acid Research. 1984. 12:8711-8721) that includes a plant gene expression cassette flanked by the left and right border sequences from the Ti plasmid of *Agrobacterium tumefaciens.* A plant gene expression cassette consists of at least two genes – a selection marker gene and a plant promoter regulating the transcription of the cDNA or genomic DNA of the trait gene. Various selection marker genes can be used including the Arabidopsis gene encoding a mutated acetohydroxy acid synthase (AHAS) enzyme (US patents 57673666 and 6225105). Similarly, various promoters can be used to regulate the trait gene to provide constitutive, developmental, tissue or environmental regulation of gene transcription. In this example, the 34S promoter (GenBank Accession numbers M59930 and X16673) was used to provide constitutive expression of the trait gene.

Canola seeds were surface-sterilized in 70% ethanol for 2 min., and then in 30% Clorox with a drop of Tween-20 for 10 min, followed by three rinses with sterilized distilled water. Seeds were then germinated in vitro 5 days on half strength MS medium without hormones, 1% sucrose, 0.7% Phytagar at 23°C, 16 hr. light. The cotyledon petiole explants with the cotyledon attached were excised from the *in vitro* seedlings, and inoculated with Agrobacterium by dipping the cut end of the petiole explant into the bacterial suspension. The explants were then cultured for 2 days on MSBAP-3 medium containing 3 mg/l BAP, 3% sucrose, 0.7% Phytagar at 23C, 16 hr light. After two days of co-cultivation with Agrobacterium, the petiole explants were transferred to MSBAP-3 medium containing 3 mg/l BAP, cefotaxime,

carbenicillin, or timentin (300 mg/l) for 7 days, and then cultured on MSBAP-3 medium with cefotaxime, carbenicillin, or timentin and selection agent until shoot regeneration. When the shoots were 5 – 10 mm in length, they were cut and transferred to shoot elongation medium (MSBAP-0.5, containing 0.5 mg/l BAP). Shoots of about 2 cm in length were transferred to the rooting medium (MS0) for root induction.

Samples of the primary transgenic plants (T0) were analyzed by PCR to confirm the presence of T-DNA. These results were confirmed by Southern hybridization in which DNA is electrophoresed on a 1% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics). The PCR DIG Probe Synthesis Kit (Roche Diagnostics) is used to prepare a digoxigenin-labelled probe by PCR, and used as recommended by the manufacturer.

The transgenic plants were then evaluated for their improved stress tolerance according to the method described in Example 5. Tolerant plants had higher seed yields, maintained their stomatal aperture and showed only slight changes in osmotic potential and proline levels, whereas the susceptible non-transgenic control plants closed their stomata and exhibited increased osmotic potential and proline levels.

Tolerance of salinity and cold were measured using methods as described in the previous example 5. Plants that had tolerance to salinity or cold had higher seed yields, photosynthesis and dry matter production than susceptible plants.

Example 10

Engineering stress-tolerant corn plants by over-expressing glutaredoxin or thioredoxin genes.

Transformation of maize (*Zea Mays L.*) is performed with a modification of the method described by Ishida et al. (1996. Nature Biotech 14745-50). Transfromation is genotype-dependent in corn and only specific genotypes are amenable to transformation and regeneration. The inbred line A188 (University of Minnesota) or hybrids with A188 as a parent are good sources of donor material for transformation (Fromm et al. 1990 Biotech 8:833-839), but other genotypes can be used successfully as well. Ears are harvested from corn plants at approximately 11 days after pollination (DAP) when the length of immature embryos is about 1 to 1.2 mm. Immature embryos are co-cultivated with *Agrobacterium tumefaciens* that carry "super binary" vectors and transgenic plants are recovered through organogenesis. The super binary vector system of Japan Tobacco is described in WO patents WO94/00977 and WO95/06722. Vectors were constructed as described. Various selection marker genes can be used including the maize gene encoding a mutated acetohydroxy acid synthase (AHAS) enzyme (US patent 6025541). Similarly, various promoters can be used to regulate the trait gene to provide constitutive, developmental, tissue or environmental regulation of gene transcription. In this example, the 34S promoter (GenBank Accession numbers M59930 and X16673) was used to provide constitutive expression of the trait gene.

Excised embryos are grown on callus induction medium, then maize regeneration medium, containing imidazolinone as a selection agent. The Petri plates are incubated in the light at 25°C for 2-3 weeks, or until shoots develop. The green shoots are transferred from each embryo to maize

rooting medium and incubated at 25°C for 2-3 weeks, until roots develop. The rooted shoots are transplanted to soil in the greenhouse. T1 seeds are produced from plants that exhibit tolerance to the imidazolinone herbicides and which are PCR positive for the transgenes.

The T1 transgenic plants were then evaluated for their improved stress tolerance according to the method described in Example 5. The T1 generation of sincle locus insertions of the the T-DNA will segregate for the transgene in a 3:1 ratio. Those progeny containing one or two copies of the transgene are tolerant of the imidazolinone herbicide, and exhibit greater tolerance of drought stress than those progeny lacking the transgenes. Tolerant plants had higher seed yields, maintained their stomatal aperture and showed only slight changes in osmotic potential and proline levels, whereas the susceptible non-transgenic control plants closed their stomata and exhibited increased osmotic potential and proline levels. Homozygous T2 plants exhibited similar phenotypes.

Tolerance of salinity and cold were measured using methods as described in the previous example 5. Plants that had tolerance to salinity or cold had higher seed yields, photosynthesis and dry matter production than susceptible plants.

Example 11

Engineering stress-tolerant wheat plants by over-expressing glutaredoxin or thioredoxin genes.

Transformation of wheat is performed with the method described by Ishida et al. (1996 Nature Biotech. 14745-50). The cultivar Bobwhite (available from CYMMIT, Mexico) is commonly used in transformation. Immature embryos are co-cultivated with *Agrobacterium tumefaciens* that carry "super binary" vectors, and transgenic plants are recovered through

organogenesis. The super binary vector system of Japan Tobacco is described in WO patents WO94/00977 and WO95/06722. Vectors were constructed as described. Various selection marker genes can be used including the maize gene encoding a mutated acetohydroxy acid synthase (AHAS) enzyme (US patent 6025541). Similarly, various promoters can be used to regulate the trait gene to provide constitutive, developmental, tissue or environmental regulation of gene transcription. In this example, the 34S promoter (GenBank Accession numbers M59930 and X16673) was used to provide constitutive expression of the trait gene.

After incubation with Agrobacterium, the embryos are grown on callus induction medium, then regeneration medium, containing imidazolinone as a selection agent. The Petri plates are incubated in the light at 25°C for 2-3 weeks, or until shoots develop. The green shoots are transferred from each embryo to rooting medium and incubated at 25°C for 2-3 weeks, until roots develop. The rooted shoots are transplanted to soil in the greenhouse. T1 seeds are produced from plants that exhibit tolerance to the imidazolinone herbicides and which are PCR positive for the transgenes.

The T1 transgenic plants were then evaluated for their improved stress tolerance according to the method described in the previous example 5. The T1 generation of single locus insertions of the the T-DNA will segregate for the transgene in a 3:1 ratio. Those progeny containing one or two copies of the transgene are tolerant of the imidazolinone herbicide, and exhibit greater tolerance of drought stress than those progeny lacking the transgenes. Tolerant plants had higher seed yields, maintained their stomatal aperture and showed only slight changes in osmotic potential and proline levels, whereas the susceptible non-transgenic control plants closed their stomata and exhibited increased osmotic potential and proline levels. Homozygous T2 plants exhibited similar phenotypes.

Legend

Figure 1: The glutaredoxin gene family showing the four subfamiles of glutaredoxin and thioredoxin coding sequences as determined by amino acid sequence homology.

Figure 2: Amino acid alignment of yeast and plant cDNA sequences of glutaredoxin subfamily 1 showing the presence of two conserved domains

Figure 3: Amino acid alignment of glutaredoxin subfamily 1 domain 1 across yeast and plant cDNA sequences. The amino acid position at the start of the alignment is shown in parenthesis.

Figure 4: Amino acid alignment of Glutaredoxin subfamily 1 domain 2 across yeast and plant cDNA sequences. The amino acid position at the start of the alignment is shown in parenthesis.

Figure 5: Amino acid alignments of yeast and plant cDNA sequences of glutaredoxin subfamily 2 showing the presence of two conserved domains.

Figure 6: Amino acid alignment of glutaredoxin subfamily 2 domain 1 across yeast and plant cDNA sequences.

Figure 7: Amino acid alignment of Glutaredoxin subfamily 2 domain 2 across yeast and plant cDNA sequences.

Figure 8: Amino acid alignments of yeast and plant cDNA sequences of glutaredoxin subfamily 3 showing the presence of two conserved domains.

Figure 9: Amino acid alignment of glutaredoxin subfamily 3 domain 1 across yeast and plant cDNA sequences.

Figure 10: Amino acid alignment of Glutaredoxin subfamily 3 domain 2 across yeast and plant cDNA sequences.

Figure 11: Amino acid alignments of yeast and plant cDNA sequences of glutaredoxin subfamily 4 showing the presence of two conserved domains.

Figure 12: Amino acid alignment of glutaredoxin subfamily 4 domain 1 across yeast and plant cDNA sequences.

Figure 13: Amino acid alignment of glutaredoxin subfamily 4 domain 2 across yeast and plant cDNA sequences.